A NOVEL APPROACH TO DEVELOP PREDICTIVE BIOMARKERS

Prediction of response to anti-EGFR therapy in a large panel of patient-derived colorectal cancer xenograft models

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ABSTRACT

Over the last ten years two major trends have influenced drug development. Instead of cytotoxic, non-specific therapies a variety of targeted small molecule agents and monoclonal antibodies have been developed. In parallel, higher attention has been paid to the identification and validation of predictive markers, which allow the stratification of patients prior to the treatment. Hundreds of targeted cancer drugs have been developed, but only a few have been approved. Despite these two trends, there is still a high rate of failure in translating promising preclinical data obtained with cancer cell lines and xenograft models derived from cancer cell lines into positive clinical phase II and phase III data. The goal of the scientific community is to develop novel targeted therapies for the majority of solid tumours with unmet clinical need using better preclinical tumour models and to link these with the ability to discover effective predictive markers that allow efficient stratification of responders from non-responders.

To bridge the gap between preclinical and clinical development a panel of 133 patients-derived CRC xenografts of all four UICC stages was established. As a proof of concept, an efficacy of three drugs approved for the treatment of CRC: cetuximab, bevacizumab and oxaliplatin was tested in the subset of 67 xenograft models derived of chemonaive CRC patients. In the treatment experiment with cetuximab monotherapy an objective response rate of 27% translating into 18 responders and 49 non-responders was obtained. This high response rate allowed the use the available tumour tissues for evaluating molecular markers for predicting response to the anti-EGFR antibody in the xenograft models. The accuracy of three mutation markers including KRAS, BRAF and PIK3CA was investigated in combination with RNA expression levels of two EGFR ligands – amphiregulin and epiregulin. Novel predictive markers panels based on gene expression profiling of mRNA and microRNA were also tested and compared with the established biomarkers. Successful reconstruction of the clinical situation in the panel of xenograft models proves their potential for future use in testing of novel anti-cancer drugs. Moreover, their broad molecular characterization allows the simultaneous development of predictive biomarkers along with the testing of novel cancer drugs.

My Ph.D. thesis contributes to the development of novel preclinical tools for cancer drug testing and discovery of accompanying predictive markers for patient selection in colorectal cancer.

EIN NEUARTIGER ANSATZ ZUR ENTWICKLUNG VON PRÄDIKTIVEN BIOMARKERN

Vorhersage der Reaktion auf anti-EGFR-Therapie in einem großen Panel von Patienten-stammenden Darmkrebs Xenografts

ZUSAMMENFASSUNG

Die Entwicklung von Medikamenten zur Behandlung von Krebserkrankungen wurde in den letzten zehn Jahren von zwei großen Trends beeinflusst. Statt zytotoxischer, unspezifischer Therapien wie bisher, wurden verschiedene zielgerichtete kleine Moleküle sowie monoklonale Antikörper entwickelt. Zudem verstärkten sich die Bemühungen in der Entwicklung und Validierung prädiktiver Biomarker (z. B. Marker-Signaturen), die die Stratifizierung von Patienten vor der Behandlung ermöglichen. Obwohl hunderte von zielgerichteten Krebsmedikamenten entwickelt wurden, erreichten nur wenige davon eine Zulassung.

Grund hierfür ist die noch hohe Ausfallrate bei der „Übersetzung" von vielversprechenden präklinischen Daten, die mit Krebs-Zelllinien und Xenograft-Modellen von Krebs-Zelllinien erlangt wurden, in positive klinische Phase II- und Phase III-Daten. Das Ziel der jüngsten Forschung ist es, neue zielgerichtete Therapien mit Hilfe von verbesserten präklinischen Tumormodellen für die Mehrzahl der soliden Tumore mit ungedecktem klinischem Bedarf zu entwickeln. Mit Hilfe dieser Modelle sollen effektive und prädiktive Marker zur effizienten Unterscheidung von Responder und Nicht-Responder entwickelt werden.

Um die Lücke zwischen präklinischer und klinischer Entwicklung zu überbrücken, wurde unter Verwendung von Tumorgewebe von 133 Patienten ein Panel an CRC-Xenograftmodellen aller vier UICC-Stadien etabliert. Zur Überprüfung dieses Modells wurde die Wirksamkeit von drei Medikamenten getestet, die zur Behandlung von CRC zugelassen sind: Cetuximab, Bevacizumab und Oxaliplatin. Für dieses Experiment benutzten wir 67 Xenograft-Modelle, die aus chemonaiven CRC-Patienten entwickelt wurden. Die Behandlung mit einer Cetuximab-Monotherapie ergab eine objektive Ansprechrate von 27% (18 Responder und 49 Non-Responder). Dank dieser hohen Ansprechrate der Therapie, konnten wir das verfügbare Tumorgewebe für die Beurteilung molekularer Marker und zur Vorhersage der Reaktion auf die anti EGFR-Antikörper in den Xenograft-Modellen verwenden.

Wir untersuchten die Genauigkeit von drei Mutations-Markern (KRAS, BRAF und PIK3CA), und kombinierten den Mutations-Status dieser drei Gene mit RNA-Expressionsdaten von zwei

Liganden der epidermalen Wachstumsfaktor-Rezeptor-Familie (Amphiregulin und Epiregulin). Weiterhin wurden neue prädiktive Marker-Kombinationen, basierend auf einer mRNA- und microRNA- Expressionsanalyse ermittelt.

Aufgrund der erfolgreichen Nachbildung der klinischen Situation in unserem Panel von Xenograft-Modellen, zeigt dieser Modellansatz vielversprechendes Potenzial für eine zukünftige Anwendung in der Erprobung neuartiger Krebsmedikamente. Darüber hinaus ermöglicht die breite molekulare Charakterisierung der Modelle beides: eine Entwicklung prädiktiver Biomarker und eine Erprobung neuartiger Krebsmedikamente.

Meine Dissertation leistet einen Beitrag zur Entwicklung neuartiger präklinischer Modelle zur Testung von Krebsmedikamenten einerseits und zur Entwicklung von prädiktiven Markern zur Optimierung der Behandlungsstrategie von Darmkrebspatienten andererseits.

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List of Abbreviations and Acronyms

5-FU	- 5-fluorouracil
A	- adenine
AJCC	- American Joint Committee on Cancer
APC	- adenomatous polyposis coli
app.	- approximately
AREG	- amphiregulin
aRNA	- antisense ribonucleic acid
ATP	- adenosine-5'-triphosphate
AUC	- area under the curve
BRAF	- V-raf murine sarcoma viral oncogene homolog B1
BSA	- bovine serum albumine
CAPOX	- capecitabine in combination with oxaliplatin
cDNA	- complementary DNA
C	- cytosine
CEA	- carcinoembryonic antigen
CI	- confidence interval
CIN	- chromosomal instability
COSMIC	- catalogue of somatic mutations in cancer
CRC	- colorectal cancer
CTNNβ1	- catenin (cadherin-associated protein), beta 1
CR	- complete response
Ct	- threshold cycle
DCC	- deleted in colorectal carcinoma
DMSO	- dimethyl sulfoxide
DNA	- deoxyribonucleic acid
dsDNA	- double-stranded deoxyribonucleic acid
DSF	- disease-free survival
DUSP6	- dual specificity phosphatase 6; negatively regulate members of the MAPK superfamily
EREG	- epiregulin
et al.	- and others
FAP	- familial adenomatous polyposis
FARMS	- factor analysis for robust microarray summarization
FBXW7	- F-box/WD repeat-containing protein 7
FFPE	- formalin-fixed paraffin-embedded
FN	- false negatives
FOBT	- fecal occult blood test
FP	- false positives
FOLFIRI	- leucovorin (folinic acid), 5-FU, and irinotecan
FOLFOX	- leucovorin (folinic acid), 5-FU, and oxaliplatin
G	- guanine
GAPDH	- glyceraldehyde 3-phosphate dehydrogenase
GEP	- gene expression profiling
GCC	- guanylyl cyclase C
gDNA	- genomic DNA
GDP	- guanosine diphosphate

GTP	-	guanosine triphosphate
GTPase	-	hydrolyze guanosine triphosphate (GTP)
H&E	-	haematoxylin and eosin
HER-2	-	human epidermal growth factor receptor 2
HK	-	housekeeping genes
HNPCC	-	hereditary non-polyposis colorectal cancer
iFOBT	-	immunological fecal occult blood test
IHC	-	immunohistochemistry
IVT	-	in vitro transcription
KRAS	-	Kirsten rat sarcoma viral oncogene homolog
LNR	-	lymph node ratio
LOH	-	loss of heterozygosity
mAb	-	monoclonal antibody
MAPK	-	mitogen-activated protein kinase
mCRC	-	metastatic colorectal cancer patients
MEK	-	mitogen-activated protein kinase kinase
MGB	-	minor groove binder
miR/miRNA	-	microRNA
MLH1	-	MutL homolog 1
MMP7	-	matrix metalloproteinase 7
MMR	-	mismatch repair system
mRNA	-	messenger RNA
MSI	-	microsatellite instability
MSKK	-	Molecular Signatures in Colorectal Cancer
MTD	-	maximum tolerated dose
NMRI	-	Naval Medical Research Institute.
NOD/SCID	-	Non-obese diabetic /Severe Combined Immunodeficiency
NPV	-	negative predictive value
NR	-	non-responders
NRAS	-	neuroblastoma RAS viral (v-ras) oncogene homolog
NSCLC	-	non-small cell lung cancer
Nt	-	nucleotide
NTC	-	non-template control
OR	-	odds ratio
ORR	-	overall response rate
OS	-	overall survival
PCC	-	Pearson correlation coefficient
PCR	-	polymerase chain reaction
PFS	-	progression-free survival
PI3K	-	phosphoinositide 3-kinase
PIK3CA	-	phosphoinositides-3-kinase, catalytic, alpha polypeptide
PMS1/2	-	postmeiotic segregation increased 1/2
PPV	-	positive predictive value
PR	-	partial response
PTEN	-	phosphatase and tensin homolog
PTPRF	-	receptor-type tyrosine-protein phosphatase F
QUASAR	-	quick and simple and reliable
R	-	responder
RESICT	-	response evaluation criteria in solid tumours

RIN	-	RNA integrity number
RNA	-	ribonucleic acid
ROC	-	receiver operating characteristic
RPLP0	-	60S acidic ribosomal protein P
RT	-	room temperature
RVS	-	retrospective validation study
(S+)	-	sensitivity
(S-)	-	specificity
SLC26A3	-	solute carrier family 26, member 3
SMAD2/3/4	-	SMAD family member 2/3/4
SNPs	-	single nucleotide polymorphisms
SOC	-	standard of care
T/C ratio	-	treated to control ratio
UBC	-	ubiquitin C
VDAC2	-	voltage-dependent anion-selective channel protein 2
T	-	thymine
TGF-ß	-	transforming growth factor beta
TGFBR2	-	transforming growth factor, beta receptor II
TN	-	true negatives
TP	-	true positives
TP53	-	tumour protein 53
VEGFA	-	vascular endothelial growth factor A
UICC	-	Union for International Cancer Control
WT	-	wild-type
XELIRI	-	a combination of capecitabine and irinotecan

Chapter 1

Introduction

1.1. BASIC FACTS ABOUT COLORECTAL CANCER

Colorectal cancer (CRC) forms in the epithelial tissue of the large intestine, which includes the caecum, colon, rectum, and anal canal (Fig.1.1; Tab.S1). It is the third most common cancer in man and the second most common cancer in women with an estimated 1.2 million new cases diagnosed each year worldwide. In fact 10% of all cancer patients suffer from colorectal cancer. CRC is the fourth leading cause of cancer-related deaths. Approximately 600.000 incidents every year account for 8% of all cancer deaths (Jemal et al., 2011).

Germany is among the countries with the highest incidence rate of colorectal cancer with more than 71.000 new occurrences annually (71/100.000 in males; 50/100.000 in females), and the highest mortality rates with app. 30.000 death cases among CRC patients each year (Sieg and Friedrich, 2009).

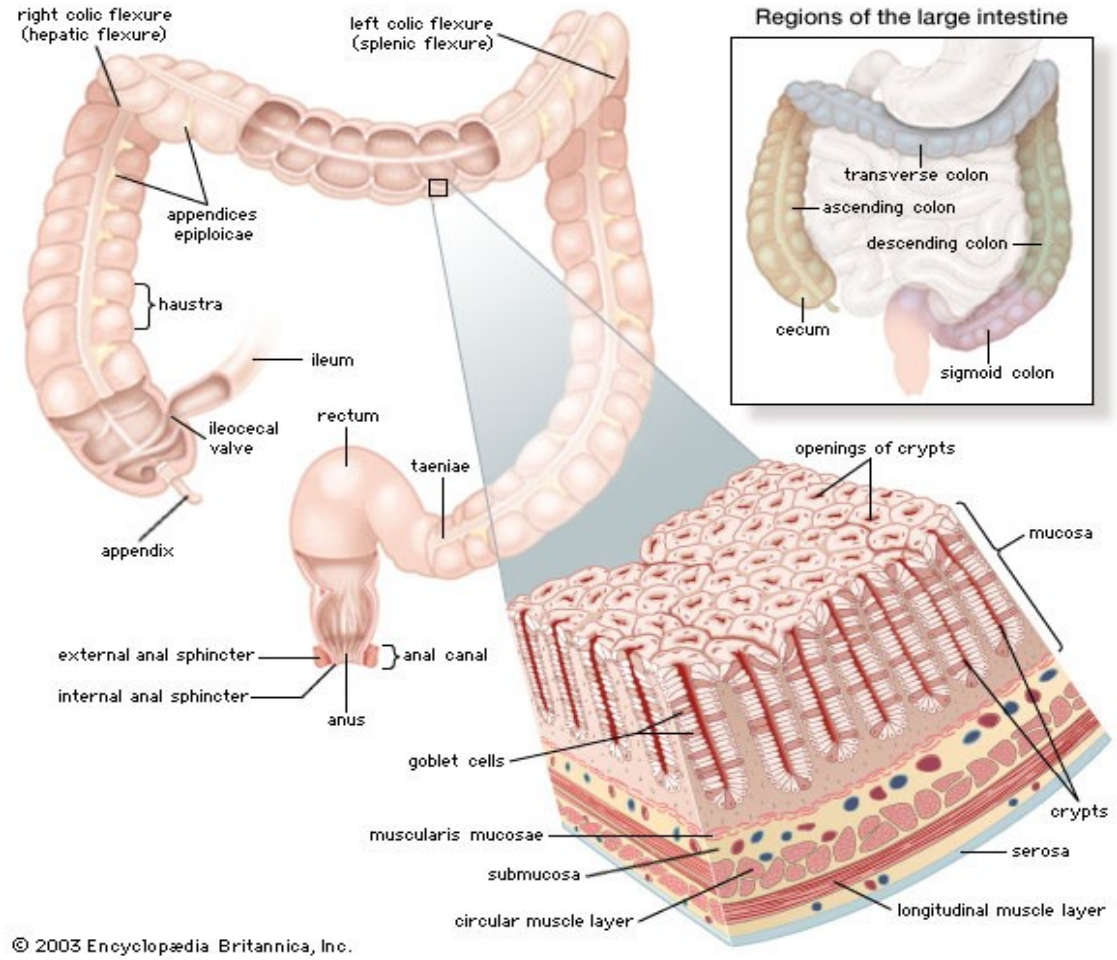


Fig.1.1. *Anatomy and histology of the large intestine: ascending, transverse, descending, sigmoid colon, and rectum (Encyclopedia Britannica, 2003)*

Colorectal cancer is traditionally divided into two categories: sporadic and familial CRC. Sporadic colorectal cancer occurs in people who have no family history of the disease and familial colorectal cancer is a hereditary syndrome. These are separate diseases both on phenotypic and molecular levels. The majority of the CRC patients (app. 75%) suffer from sporadic colorectal cancer and all the samples used in this study origin from the patients with non-hereditary CRC. The sporadic type of this disease is therefore a focus of this work. A number of patients, however, develop CRC as a result of an inherited genetic condition. The most common hereditary CRC predisposing syndrome is hereditary non-polyposis colorectal cancer (HNPCC/Lynch syndrome) that accounts for app. 3 % of all CRC cases. The familial adenomatous polyposis (FAP) syndrome accounts for additional 1%. Other, less frequent colon cancer syndromes include Peutz-Jeghers syndrome (PJS), Juvenile polyposis syndrome (JPS), hereditary mixed polyposis syndrome (MHAP) and Cowden's syndrome. The causes underlying the majority of remaining familial CRCs cases are not yet understood (Migliore et al., 2011).

1.2. CLINICAL BASIS OF COLORECTAL CANCER

1.2.1. Clinical Staging

At the moment of diagnosis colorectal cancer patients are classified in one out of five clinical stages (0-IV) according to the guidelines of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). Clinical stages describe the extent of the cancer's spread in the body (Fig.1.2) and are based on the results of the physical exam, biopsies, imaging tests (computed tomography, magnetic resonance imaging scan, x-rays, positron emission tomography, endoscopic or abdominal ultrasound) and results of the surgery followed by histopathological diagnosis. In the earliest stage 0 the tumour is small in size and limited to the inner layer (mucosa) of the colon or rectum (carcinoma in situ). In stage I the cancer has grown through the muscularis mucosa into the submucosa or muscularis propria. Stage II colorectal cancers are larger and extend through the muscular wall of the colon or rectum. In stage III the tumour has spread to one or more nearby lymph nodes. Finally, in the most advance stage IV, the tumour metastasized to distant organs, such as liver or lungs (for details, see Tab.S2).

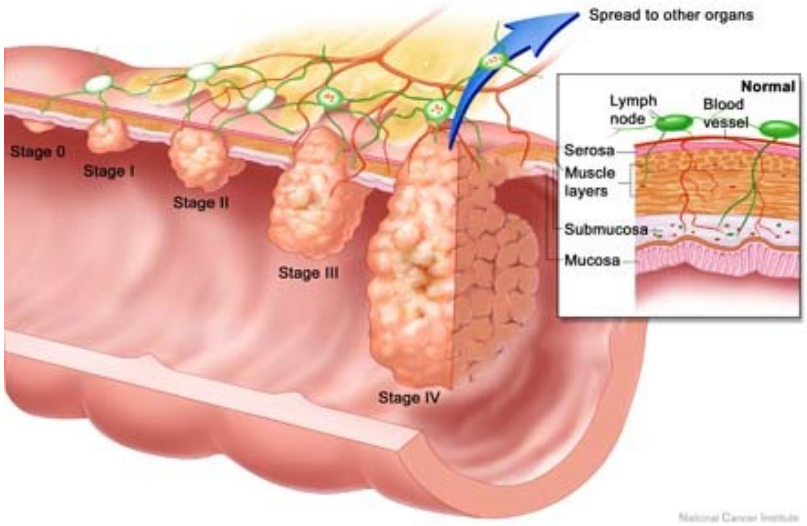


Fig.1.2. *Spread of the tumour through and outside the layers of intestinal wall in CRC stages 0-IV (image from the National Cancer Institute www.cancer.gov)*

1.2.2. Treatment of CRC Patients

Clinical stage of colorectal cancer is the foundation on which the treatment recommendations are based. For colorectal cancers that have not spread to distant sites (stages 0-III), surgery is usually the initial treatment to remove the tumour mass.

In stage III CRC adjuvant (additional) chemotherapy is generally recommended. The FOLFOX (folinic acid, 5-fluorouracil, and oxaliplatin) regimen is still the most common

chemotherapy combination. Orally available 5-fluorouracil (5-FU), capecitabine, alone or in combination with oxaliplatin (CAPOX) has lately been shown to be equivalent to the FOLFOX regimen and is more convenient. Further chemotherapy regimens relevant to current clinical practice are based on irinotecan. This topoisomerase inhibitor is used typically in combination with other chemotherapeutic drugs: folinic acid and 5-FU (FOLFIRI) or with capecitabine (XELIRI). There are no clear recommendations concerning adjuvant treatment in stage II CRC. Only stage II patients of high-risk group are, therefore, selected for the postoperative adjuvant therapy.

Although colon and rectal cancer occur anatomically in continuity and have similar histopathological features, there is uncertainty regarding the benefits from the adjuvant chemotherapy regimens established for colon cancer patients in the group of the patients with rectal cancer. For patients with rectum carcinoma of stage II and III adjuvant treatment with 5-FU and radiation, followed by resection of the reduced tumour mass, represents a valuable alternative to the traditional resection of the primary tumour followed by adjuvant chemotherapy. However, recent data demonstrate high long term toxicity due to the radiation therapy which needs to be addressed in the future.

The situation for patients with metastatic stage IV (mCRC) is much more complex and cannot be described in detail in this chapter. For short, in the majority of cases there is no cure for these patients despite many different treatment options (Van Cutsem et al., 2010). The clinical cases, in which it is possible to completely remove the primary tumour in the colon or rectum and the metastases, are very rare. The majority of patients have a metastatic disease that initially is not suitable for surgery. In such cases neoadjuvant therapy would be applied to reduce the size of metastases and allow the surgery. Treatment of the metastatic disease in CRC patients is extremely challenging as the disease in its most advanced stadium often progresses very quickly. Furthermore it is often not known which patient will benefit from the treatment. Different therapy options are available and can be grouped into first-, second and third-line therapy. In case of a patient who does not respond to the standard therapy, another treatment strategy is applied (Edwards et al. 2012) (Tab.1.1.). Treatment regimens for mCRC include not only cytotoxic chemotherapy but also targeted monoclonal antibodies (mAb): bevacizumab, cetuximab and panitumumab.

Tab.1.1. *A simplified model of the treatment options for the patients with stage IV colorectal patients according to the previously administered therapy; KRAS mutation status not considered; mAb - monoclonal antibody; 5-FU - 5 fluorouaracil; IRI - irinotecan*

Patients / Therapy	Chemonaive			Pre-treated	
1st line	5-FU + leucovorin /FOLFOX/FOLFIRI /FOLFOXIRI /capecitabine /CAPOX	cetuximab/ panitumumab + FOLFOX/ FOLFIRI	bevacizumab + FOLFOX/ FOLFIRI/ CAPOX, 5-FU/leucovorin or capecitabine	cetuximab/ panitumumab	cetuximab + IRI
2nd line	cetuximab/ panitumumab +/- FOLFIRI	cetuximab + IRI	bevacizumab + FOLFOX	alternative mAb	
3rd line	alternative mAb +/- FOLFOX/FOLFIRI			alternative mAb	

1.2.2.1 Monoclonal antibodies in the treatment of metastatic CRC

Both, cetuximab - a mouse-human chimeric monoclonal antibody and panitumumab - a fully human monoclonal antibody bind to the epidermal growth factor receptor (EGFR) which is one of the major growth factors in tumourigenesis and disease progression. These antibodies are used in first-, second-, and third-line treatment of mCRC (Fig.1.3B). Cetuximab and panitumumab bind to the extracellular domain of the EGFR and through tertiary structure change of the EGF receptor prevent the binding of the natural ligands: EGF, TGF- α, amphiregulin (AREG), epiregulin (EREG), heparin-binding EGF-like growth factor (HB-EGF), and β-cellulin to the receptor. Thus, the binding of cetuximab and panitumumab prevent ligand-induced activation. By blocking signal transduction downstream of EGFR, cetuximab and panitumumab inhibit cell growth, proliferation, survival as well as angiogenesis, cell migration and adhesion (van Krieken et al., 2008). Mechanism of action of bevacizumab, a mAb which is also approved in first line, second line and third line therapy, is completely different. Bevacizumab targets the most potent proangiogenic factor identified to date – the vascular endothelial growth factor (VEGF). During angiogenesis new tumour vessels are formed. They are necessary to supply the tumour with nutrients and oxygen but also allow tumour cells to enter the circulation, infiltrate distant organs and establish metastases. By binding to VEGF, bevacizumab prevents VEGF from binding to its natural receptor and thus inhibits stimulation of the intracellular transduction of proangiogenic signals (Tol and Punt, 2010; Venook, 2005).

1.2.3. Survival rates in colorectal cancer patients

In the past 15 years new treatment options for patients with colorectal cancer evolved. In 1995 5-fluorouracil used to be the sole treatment option for local defined CRC and for advanced disease. Significant advances have been made owing primarily to the improvement of surgical methods and pharmaceutical interventions. For example, resection of single liver metastases and lung metastases has improved dramatically

leading to prolonged survival rates for these patients. However, only a small subgroup benefits from the operations (Segal and Saltz, 2009). As described in the previous chapter, new antibody-based treatment options and chemotherapeutic agents have been introduced. Beside improvements in the progression-free survival (PFS) of patients, one of the two common end-points of clinical trials, the long-term overall survival (OS) rates remain unsatisfactory, particularly in patients with locally advanced (stage III), or metastatic (stage IV) CRC, who represent about 30% and 15% of all CRC patients, respectively.

The 5-year survival rate for CRC patients is greater than 90% when tumours are detected at a localized early stage I. The 5-year survival rate refers to the percentage of patients who survive at least 5 years after cancer diagnosis. It decreases to app. 80% in patients diagnosed with stage II CRC. If the tumour affects local lymph node (stage III patients) the 5-year survival rate is only 50-69% despite today's standard of care which is surgical removal of the tumour followed by adjuvant therapy. Once the tumour has spread to distant organs (stage IV patients), a chance to survive the next five years drops to 4-12% despite the various new treatment options (O'Connell et al., 2004; Oh et al., 2007). This indicates that the approval of targeted antibodies has not led to an increase in overall survival, but rather only to a benefit in terms of progression-free survival. Although up-to-date long-term survival trends show significant increases, there is a large unmet clinical need in advanced, metastatic CRC and for the 20% of patients in stage II and 40% of patients in stage III, who will progress despite the standard of care (SOC).

There are several reasons for a poor outcome in a significant number of patients. Lack of effective early detection programs in many countries hinders early diagnosis of CRC. Because of complexity and heterogeneity of individual tumours, there are also no accurate molecular markers that can be applied for prognostic purposes. Moreover, despite the variety of treatment options, the response rates remain poor and there are no clear guidelines to select patients who may benefit from the therapy. These aspects strongly support, but not facilitate, the discovery of cancer biomarkers.

1.3. BIOMARKERS OF COLORECTAL CANCER

According to the US National Institutes of Health's (NIH) a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Naylor, 2003). Statistical measures used to evaluate the accuracy of the biomarker are sensitivity (S+) and specificity (S-). Sensitivity relates to the ability of the test to detect positive results. It is the proportion of true positives (TP), which are correctly identified

as such, among true positives and false negatives (FP) (S+= TP/(TP+FN)). Specificity evaluates a test's ability to identify negative results. Specificity measures the ratio of true negatives (TN), which are correctly identified among true negatives and false positives (FP) (S-=TN/(TN+FP)).

There are diagnostic, prognostic and predictive biomarkers, grouped with respect to their clinical applications. Diagnostic, or early-detection biomarkers, are measurable parameters that indicate the presence of cancer in the body of a patient. Diagnostic biomarkers can be used for early detection or screening of cancer in a population. Another group - prognostic biomarkers, is applied to indicate how the disease will develop in an individual case. Standard clinicopathological risk assessment is based on the staging system. Stage describes the severity of the cancer and indicates distinct treatment recommendations, but even extensive analysis of clinicopathological determinants is not always sufficient to predict a patients' outcome. Altogether a recurrence rate of app. 40-50% is observed in CRC indicating the lack of accurate determinants whether to recommend additional treatment.

1.3.1 Predictive Biomarkers

While diagnostic and prognostic biomarkers are not yet ready for routine use due to challenges in their clinical validation for early disease detection and long-term survival improvement, a third group of predictive biomarkers has established position in the clinical routine of colorectal cancer. Predictive biomarkers are used to assess, prior to the treatment, whether the patient is likely to respond to this particular agent. The purpose of a patients' stratification is, on the one hand, to increase overall response rate to the therapeutic agent and, on the other hand, to decrease unnecessary toxic side effects and treatment costs. Predictive biomarkers are particularly important in patients of stage IV CRC for whom the consequences of the administration of ineffective treatment are most dramatic. A chemotherapy with the most commonly applied regimens of FOLFOX and FOLFIRI achieves relatively high response rates in 40% to over 50% (Douillard et al., 2000; de Gramont et al., 2000) of CRC patients due to their overall non-specific cytotoxic effect. In contrary, the molecular mechanism of action of targeted therapies, particularly mAb, used in the treatment of advanced colorectal cancer is limited to the selected pathway. Any alterations in that pathway may affect efficacy of mAb, therefore significantly less patients benefit from the agents directed against a particular target.

1.3.2 Biomarkers Discovery and Targeted Therapies

Such situation is observed in the treatment of mCRC with monoclonal antibodies: cetuximab and panitumumab. Cetuximab was the first immunotherapy to treat colorectal cancer patients. This mAb was approved in 2004 beside the low overall response rates (ORR) observed in 11% of patients treated with single-agent cetuximab and 23% of patients treated with cetuximab in combination with irinotecan (Cunningham et al., 2004). Approval of panitumumab in 2006 was based on even less optimistic efficacy data. Only 8% of the patients assigned to panitumumab responded to the treatment. Clinical trials that followed the approval of anti-EGFR therapies showed response rates between 10% and 20% when single-agents were applied to chemorefractory metastatic patients (Amado et al., 2008; Freeman et al., 2008; Karapetis et al., 2008a; Karapetis et al., 2008b), while in trials with combination therapy response rates of 20-30% were observed (Benvenuti et al., 2007; Di Fiore et al., 2007; De Roock et al., 2008). Anti-EGFR mAb as first line therapy of metastatic colorectal cancer in chemonaive patients showed complete or partial response rate (ORR) of 46-47% when tested in combination with FOLFOX and FOLFIRI (Amado et al., 2008; Freeman et al., 2008; Karapetis et al., 2008a; Van Cutsem et al., 2009).

Low response rates to anti-EGFR treatment, reported particularly in chemorefractory patients, stimulated search for biomarkers that would allow identification of potential responders and resistant cases. It took years to learn that neither the mutation status of EGFR nor the EGFR expression is an effective biomarker for predicting response. Mutation analysis of EGFR in a group of 293 colorectal cancer specimens, revealed that they are rare events in colorectal patients and are, therefore, unlikely to be responsible for the resistance toward cetuximab as only one mutation was detected in this setting (Barber et al., 2004). As well EGFR expression as detected by IHC showed no correlation with cetuximab activity (Chung et al., 2005). EGFR – a target for cetuximab and panitumumab, failed as predictive biomarker of mAbs effectiveness in colorectal cancer, therefore attention was given to the signaling pathways downstream of the EGF receptor.

1.3.4.1 Mutations in the KRAS gene

The first one who reported a link between the genetic alterations and the response to the targeted treatment was Lievre in 2006. Lievre and colleagues reported that mutations in the Kirsten rat sarcoma viral oncogene homolog (KRAS) are responsible for resistance to cetuximab (Lievre et al., 2006). Eleven of the 30 patients (37%) enrolled into the study responded to cetuximab. Out of seventeen patients with wild-type KRAS status eleven (65%) responded to cetuximab, while among the patients who carried mutations in

codon 12 or 13 of the KRAS gene no responders were observed. This association was explored by numerous population based studies (Benvenuti et al., 2007; De Roock et al., 2008; Di Fiore et al., 2007; Lievre and Laurent-Puig, 2009; Maughan et al., 2011) confirming that KRAS is a powerful predictive factor in relation to the efficacy of anti-EGFR treatment. KRAS encodes a 21-kDa signaling G-protein that is a member of the small GTPase superfamily and is activated by EGFR. It plays a role in signal transduction from the membrane to the cell's nucleus. KRAS is activated as a response to the binding of extracellular signals such as growth factors, cytokines, or hormones to the cell surface receptors (Fig.1.3A).

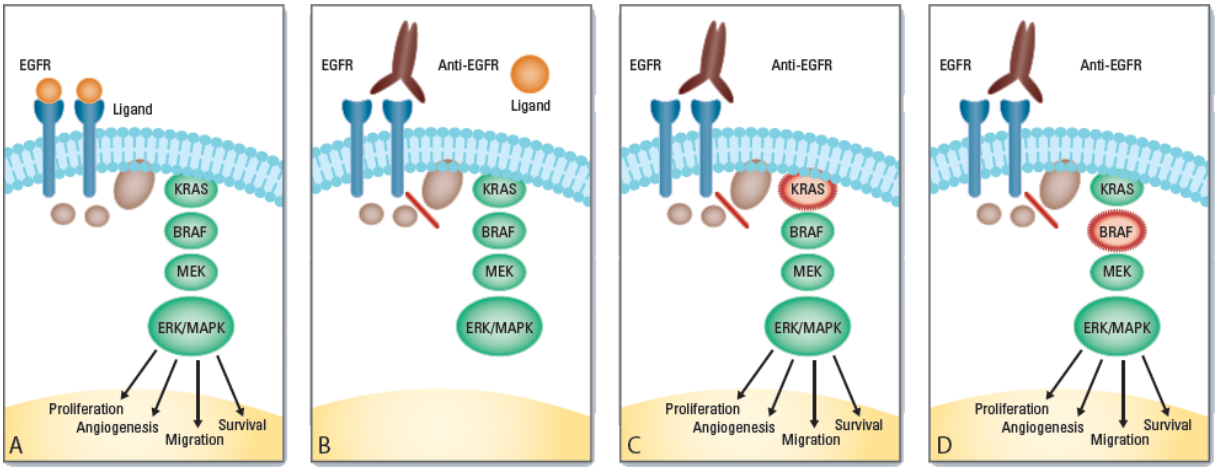


Fig.1.3. *A) EGFR pathway with its main effectors B) EGFR pathway inhibited by monoclonal antibodies that bind the extracellular domain of EGFR C) Mutations in the oncogene KRAS cause its permanent activation and stimulate signal transduction downstream of the receptor, regardless of a blocking mAb D) BRAF mutations show similar results to those in KRAS (image from: Clarient 2011 http://www.clarientinc.com)*

Oncogenic mutations in KRAS impair the hydrolysis of RAS-bound GTP to GDP. KRAS, locked in the GTP-bound state, is constitutively activated regardless of EGFR (Fig.1.3C). Administration of anti-EGFR agents in patients who harbour KRAS mutations in codon 12 and 13 is, therefore, of no benefit to the patient. Further studies confirmed the oncogenic effect of mutations in codon 61, as well as those in codon 146 (Prenen et al., 2009; Loupakis et al., 2009). Nevertheless, resistance to anti-EGFR agents observed in 80-90% of treated CRC patients could not be fully explained by mutations in the KRAS gene that are found in app. 35-45% of CRC patients (Siena et al., 2009).

1.3.4.2 Mutations in the BRAF gene

A direct downstream effector of KRAS in EGFR signaling pathway – the serine/threonine kinase V-raf murine sarcoma viral oncogene homolog B1 (BRAF) was therefore investigated as a potential biomarker. Mutation analysis conducted in 113 patients

treated either with cetuximab or panitumumab revealed that indeed not only KRAS wild-type status, but as well BRAF wild-type status is required for response to anti-EGFR therapy in metastatic colorectal cancer (Di Nicolantonio et al., 2008; Benvenuti et al., 2007;). The BRAF gene encodes a serine/threonine kinase, which phosphorylates and activates the Mitogen-activated protein kinase kinase (MEK). MEK triggers a phosphorylation of ERK, which translocates to the nucleus. There it regulates activities of several transcription factors, which induce expression of the genes required for survival and proliferation (Robinson and Cobb, 1997; Montagut and Settleman, 2009). A missense substitution at residue V600E accounts for more than 90% of all mutations in the BRAF gene. This alteration mimics phosphorylation by inserting a negatively charged residue adjacent to a phosphorylation site. The active conformation of the BRAF protein leads to significant elevation of its kinase activity. Mutated BRAF permanently stimulates signal transduction in the EGFR pathway, irrespective of the received signal (Fig.1.3D) (Davies et al., 2002; Wan et al., 2004; Garnett and Marais, 2004). BRAF mutations as mutually exclusive with KRAS mutations, account for additional 10% of resistant cases (Tie et al., 2011).

1.3.4.3 Mutations in the NRAS gene

The neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) is closely related to the KRAS gene. NRAS encodes a membrane protein that plays role in signal transduction between the Golgi apparatus and the plasma membrane. Activating mutations of this homologue of KRAS do not occur very frequently and were reported in about 2-4% of colorectal cancer patients (Bardelli and Siena, 2010; Maughan et al., 2011). NRAS mutations were proven to be associated with lack of response to cetuximab (De Roock et al., 2010a) as well as to panitumumab (Peeters M, 2012) in randomized clinical trial settings.

1.3.4.4 Mutations in the PIK3CA gene

Another gene that drew attention as a potential biomarker is the Phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA), but its role in the resistance to anti-EGFR agents remains controversial (De Roock et al., 2011; Jhawer et al., 2008; Prenen et al., 2009; Sartore-Bianchi et al., 2009b). PIK3CA is a critical component of the PI3K/Akt signaling pathway downstream of EGFR. It encodes a 110 kDa catalytic subunit of a lipid kinase. PIK3CA recruits ATP to phosphorylate 3'OH of the inositol ring of phosphoinositides and is involved in a wide variety of cellular processes such as proliferation, growth, cell survival, vesicular trafficking or cell migration (Nosho et al., 2008). Mutations in PIK3CA are found in app. 15 % of colorectal cancer patients and

often coexist with KRAS or BRAF mutations (Siena et al., 2009; Pentheroudakis et al., 2013). The majority of the mutations are reported in two hotspots in exon 9 (E542K; E545K) and one hotspot in exon 20 (H1047R). A different biologic effect of mutation in codon 9 of PIK3CA, which encodes a helical domain and mutations in exon 20, which encodes a kinase domain, has as well been suggested. It was reported that only exon 20 is a potential biomarker for resistance to anti-EGFR treatment, while mutations in exon 9 had no significant effect on the response rate (De Roock et al., 2010a).

Authors of this population-based study suggest the sequential assessment of mutations in KRAS, BRAF, NRAS and PIK3CA codon 20 for patients' selection prior to EGFR targeted therapies. According to their impact on the resistance to anti-EGFR treatment, KRAS mutations should be assessed first (36.3% response rate), BRAF second (38.4% response rate), NRAS as third (39.9% response rate) and PIK3CA exon 20 as fourth (41.2% response rate). Such mutation analysis led to the improvement of response rate to cetuximab from 24% in unselected population to 41% in quadruple (KRAS, BRAF, NRAS, and PIK3CA exon 20) negative population. App. 60-75% of the resistant cases could be recognized by mutation analysis in KRAS, BRAF, PIK3CA exon 20, and NRAS genes.

1.3.4.5 KRAS codon 13 and response to the treatment

Later on, a meta-analysis of patients enrolled in the CO.17, BOND, MABEL, EMR202600, EVEREST, BABEL, and SALVAGE clinical trials raised further doubts regarding the patients' stratification strategy. It demonstrated that some of the patients who carry KRAS mutations in codon 13, which accounts for app. 20% of all KRAS mutations, may benefit from treatment with mAb. Results of this meta-analysis suggested that patients with mutations in KRAS codon 13, previously considered as linked with resistance to anti-EGFR, are more likely to benefit from the treatment than those who carry mutations in codon 12. These findings were confirmed in vitro by comparing an effect of cetuximab on different cell lines. Both, cell line with a wild-type status of KRAS and a cell line, which harboured mutation in KRAS codon 13 (G13D) were sensitive to cetuximab, while colorectal cells that harboured mutations in KRAS codon 12 (G12V) were insensitive to cetuximab (De Roock et al., 2010b).

Mutation based predictive biomarkers in colorectal cancer turned out to be more complex than initially thought. In fact mutation status identifies those patients that are resistant to the treatment and does not indicate actual responders. Beside KRAS codon 12 and BRAF V600E mutations other hotspots linked with resistance have low frequencies and do not improve accuracy of the biomarker substantially. Increasing number of hotspots

linked with resistance and heterogeneity of tumour samples lead to technical difficulties. Moreover, conflicting data concerning some of the hotspots (e.g. in the PIK3CA exon 9, KRAS codon 13) result in challenging interpretation.

1.3.4.6 Response to anti-EGFR treatment and gene expression signatures

Another class of biomarkers based on gene expression signatures was, next to the mutation markers, under active investigation. An impact of the gene expression pattern on the ability to respond to anti-EGFR treatment was first studied on the material from metastatic colorectal carcinomas treated with cetuximab monotherapy (Khambata-Ford et al., 2007). Gene expression profiling was performed using U133A v2.0 Affymetrix GeneChip platform. The analysis revealed that in the group of KRAS wild-type patients, a high RNA expression of amphiregulin (AREG) and epiregulin (EREG) was associated with disease control under cetuximab. AREG and EREG are members of the epidermal growth factor family and ligands of EGF/TGF-alpha receptors. It was speculated that elevated gene expression of both ligands may promote tumour growth and survival via an autocrine loop. Such a loop may characterize tumours that are EGFR-dependent. That would explain particular sensitivity to an anti-EGFR agent that block the ligand-receptor interaction. These findings were confirmed by expression analysis of AREG and EREG performed by real-time PCR in 220 primary tumours derived from mCRC patients (Jacobs et al., 2009). In another real-time PCR - based study the RNA expression of 110 selected genes was analyzed in 144 KRAS wild-type primary tumour specimens from mCRC patients (Baker et al., 2011). A classifier containing AREG, EREG and two other genes, DUSP6 (which encodes dual specificity phosphatase that blocks EGFR pathway by dephosphorylation of MAPK) and SLC26A3 (which encodes an intestinal chloride ion transporter), was proposed to yield the best predictive scores.

The attention was drawn as well to the phosphatase and tensin homolog (PTEN), which is a downstream regulator of EGFR. This tumour suppressor is a key modulator of the Akt-mTOR signaling pathway. At first, lack of PTEN expression was associated with resistance to cetuximab (Frattini et al., 2007; Sartore-Bianchi et al., 2009). Nevertheless, results of the recent CAIRO 2 study denied its predictive value for response to cetuximab, neither alone, nor in combination with other markers (Tol et al., 2010).

1.4. MOLECULAR BASIS OF COLORECTAL CANCER

1.4.1 Vogelstein/Fearon Model

So far only KRAS mutations in codon 12 and 13 are approved as a predictive biomarker to select patients eligible for anti-EGFR treatment. KRAS mutations are as well one of the first alterations associated with development of colorectal cancer already included in the Vogelstein/Fearon model for the multistep genetic process of colorectal tumourigenesis. In 1990, Fearon and Vogelstein presented evidence for the formation of colorectal cancer as a process in which a number of distinct mutations of the oncogenes and tumour suppressor genes occur sequentially over time (Fig.1.4). Their general model describes molecular abnormalities that trigger dysplasia or the formation of the polyps within the epithelial cells of the mucosa followed by one or several malignant transformations and finally metastasis of the tumour to distant organs (Fearon and Vogelstein, 1990).

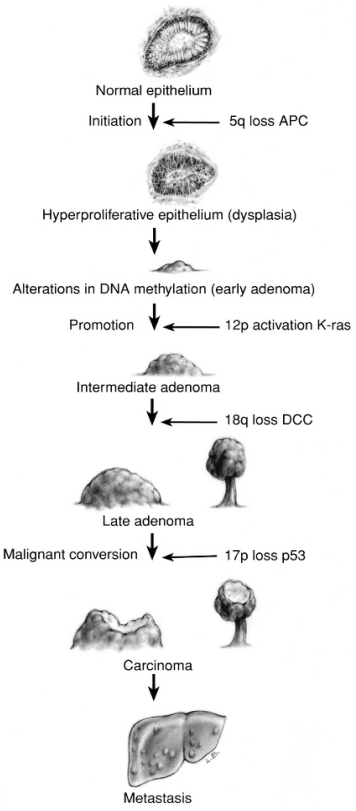


Fig.1.4. *Fearon/Vogelstein genetic model of colorectal cancer tumourigenesis (Strachan and Read, 1999; Karp and Morris, 2012)*

Intensive mutational analyses of clinical tumour samples performed by the Cancer Genome Atlas Network showed that all three key cancer genes of the model (APC, KRAS and TP53), found to be mutated in the same sample, only in app. 20% of all CRCs (The Cancer Genome Atlas Network, 2012). An intensive screening for factors involved in colorectal tumourigenesis led to identification of additional molecular alterations that are accumulated during the neoplastic process. Moreover, it was later shown that a subgroup of tumours arising from serrated polyps instead of non-serrated adenomas are mutated in the BRAF and not in the KRAS gene and follow an alternative pathway of tumourigenesis (Goldstein et al., 2003; Goldstein, 2006). The model proposed by Fearon and Vogelstein is, therefore, not representative for the majority of colorectal tumours.

Over time a more complex model of the genetic events in colorectal cancer along the adenoma – carcinoma sequence was proposed (Fig.1.5). It was enriched with a variety of factors that were identified to contribute to the CRC carcinogenesis due to intensive studies on the molecular basis of colorectal cancer over the past 20 years. Mutations in the oncogenes and tumour suppressor genes are still recognized as crucial factor in the tumour development. Oncogenes and tumour suppressors most frequently mutated in colorectal cancer patients are listed in the Tab.1.2.

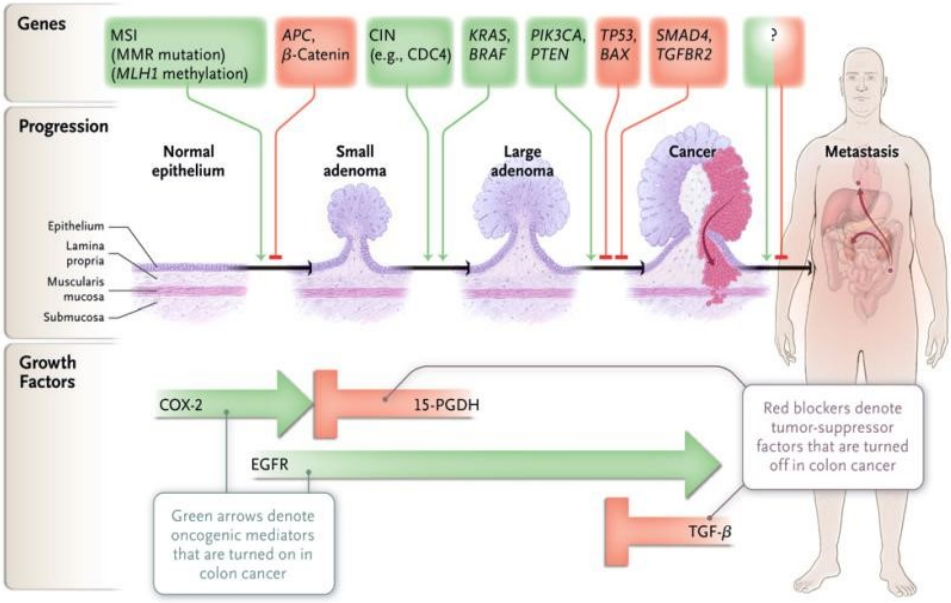


Fig.1.5. *Model of colorectal carcinogenesis by Markowitz and Bertagnolli (2009). The question mark indicates that up to date no events were identified to trigger metastasis in the progression of CRC*

Tab.1.2. *Oncogenes and tumour suppressor genes altered in colorectal cancer and their potential as biomarkers; frequencies quoted according to the Catalogue Of Somatic Mutations In Cancer (COSMIC) database; OS - overall survival, PFS - progression-free survival; resp. - responders*

Gene	Function	Mutations	%	Potential as a Biomarker
APC	Tumour suppressor gene; antagonist of the Wnt pathway that plays role in cell migration and adhesion; interacts with CTNN ß1	Wide spectrum of mutations - no hotspots of significant frequency; high fraction of InDels	35%	-
BRAF	Oncogene; serine-threonine kinase that plays role in cell growth signal transduction downstream of KRAS	Activating mutation in V600E accounts for 90% of all mutations	11%	Predictive - anti-EGFR agents; Prognostic - associated with lower OS and PFS, older age and female gender
FBXW7	Tumour suppressor gene; substrate recognition component of a SCF E3 ubiquitin-protein ligase complex; mediates the ubiquitination and degradation of target proteins	Hotspots in codons 465 and 479 account for app. 37% of reported substitutions	9%	-
CTNNß1	Oncogene; transcriptional regulator of genes involved in cell proliferation, cell cycle progression, apoptosis and in the regulation of cell adhesion	Hotspots in codons 41 and 45 account for ~25%, 36% of all mutations resp.	6%	-
KRAS	Oncogene; involved in signal transduction downstream of EGFR as a response to growth factors, cytokines, hormones	Hotspots in codon 12, 13, 61, 146 account for ~79%, 20%, 1%, 0.4% of all mutations resp.	37%	Predictive to anti-EGFR agents - approved by FDA
PIK3CA	Oncogene; lipid kinase that phosphorylates inositols; participates in cellular signaling in response to various growth factors and plays role in cells proliferation, growth, survival and migration	Hotspots in codon 542, 545, 1047 account for 18%, 33%, 26% of all mutations resp.	13%	Predictive to anti-EGFR agents - contradictory results
PTEN	Tumour suppressor gene; dual-specificity protein phosphatase and lipid phosphatase; modulator of the Akt/mTOR signaling pathway, which plays a role in cell cycle progression and cell survival	No hotspots of significant frequency	7%	Predictive to anti-EGFR agents - contradictory results
SMAD4	Tumour suppressor gene; coactivator and mediator of signal transduction by TGF-beta, transcriptional regulator	No hotspots of significant frequency	11%	-
TGFBR	Tumour suppressor gene; receptor involved in signaling pathway mediating cell cycle arrest, cell proliferation and differentiation, extracellular matrix production, immunosuppression and apoptosis	No hotspots of significant frequency	3%	-
TP53	Tumour suppressor gene; transcription factor that induces cell cycle arrest, apoptosis and DNA repair	Wide spectrum of mutations; hotspots in codon 175, 248, 273, 282 account for 6%, 5%, 5% and 4% of all mutations resp.	44%	Predictive - chemotherapy

1.4.2. Epigenetic Alterations

It turned out that cancer is more complex and results not only from the alterations at the genetic, but as well at the epigenetic level. One of the epigenetic mechanisms to initiate development and growth of colorectal cancer is aberrant methylation in the epithelial cells. It is well known that methylation of the CpG islands in the promoter region of the gene may lead to epigenetic silencing and inactivating of certain tumour suppressor genes (Issa, 2004). Moreover abberant methylation influences gene expression and leads to microsatellite instability (MSI) if DNA mismatch repair (MMR) system is affected.

1.5. INTRATUMOURAL HETEROGENEITY

A variety of molecular alterations involved in colorectal carcinogenesis was observed between individual CRC patients. The difference suggests that there are many alternative molecular pathways that lead to colorectal cancer. It has even been suggested that colorectal cancer is not a single disease, but a group of diseases with similar symptoms.

Differences in the molecular structure of the underlying genes are not only observed between CRC patients, but as well within different cellular foci of one tumour. KRAS, TP53 and chromosomal instability of 5q and 18q were studied in a group of 45 tumour specimens in order to analyze the intratumoural heterogeneity of genetic alterations (Losi et al., 2005). In each tumour 12-17 areas were distinguished depending on the tumour size. Analysis of this four genetic markers revealed that 76% of the primary tumours were heterogeneous for at least one of the investigated features. Intratumoural heterogeneity was frequent in the early stages of CRC (e.g. for KRAS mutations observed in 60% of analyzed cases; for TP53 mutations - in 70% of analyzed cases), reduced in advanced stages (KRAS in 20%; TP53 in 20%) and almost absent in colorectal metastases (KRAS - 0%; TP53 in 5%).

1.5.1 Parallel- and Clonal-Evolution Models

Intratumoural heterogeneity implicates a question of the cancer-evolution model. The traditional "clonal-evolution" model postulates that tumour development and progression is a result of clonal selection and expansion. Tumour cells that contribute a strong selective advantage supplant other tumour cells and give rise to the tumour and ultimately to metastasis (Baudot et al., 2009). In an alternative "parallel-evolution" model, it was proposed that metastasis occurs early and evolves in parallel to the growth of the primary tumour (Gray, 2003). Numerous studies addressed this question and analyzed genetic alterations of primary tumours in comparison to matched metastases (Artale et al., 2008; Molinari et al., 2009; Baldus et al., 2010). Mutation analysis of key genes such as KRAS, BRAF or PIK3CA indicated a concordance of 90-100% between

primary tumours and distant metastases. Extensive mutation analysis was conducted in 10 primary tumours and matched mestases. Out of 233 investigated somatic mutations, only seven (3%, CI:0,9=1.5-5,7%) were present in metastasis and could not be found in primary tumour from which the metastasis arose (Jones et al., 2008). These findings suggest that, in most of the cases, mutations that are necessary to initiate metastasis are already present in the primary tumour of which they derive. This is supported by the observation that most of the metastases from the same patient have identical molecular background. It is possible though, that a small population of cells within the tumour gains its capacity to metastasize due to additional mutations. Lack of concordance between primary tumours and matched metastases or different metastases from the same patient in app. 10% of analyzed patients can be explained by the parallel-evolution model. In this model the tumour may originate from only one cancer stem-cell. The tumour may also evolve independently using different mechanisms of progression within one tumour (Klein, 2009). So far, a general mutation pattern responsible for metastasis has not been identified. The heterogeneous pattern of tumour mutations suggests that multiple alternative genetic pathways exist. They lead to the formation, growth and recurrence of colorectal cancer in individual patients, and probably also within one tumour, which reflects the complexity of colorectal cancer development and progression.

1.6 CLINICAL TRIALS IN COLORECTAL CANCER

Colorectal cancer is a highly complex disease caused by a combination of modified genetic variants and aberrant epigenetic states. The heterogeneous patterns of molecular events suggest that multiple alternative genetic and epigenetic pathways to colorectal cancer exist. Moreover, differences observed within the lesion of the same tumour specimen would support the presence of intratumoural heterozygosity and intratumoural subclones. Our growing understanding about cancer resulted in the development of new treatment options. These new treatment options have, however, not significantly increased 5-year survival rate for the majority of the mCRC patients. Moreover, they are beneficial only in a very limited fraction of patients. Predictive biomarkers for patients' stratification prior to the therapy have become lately a standard procedure, but their accuracy is still not satisfying. Thus the current clinical situation stimulates a discussion about the standard approach to the clinical trial.

1.6.1 Clinical and Preclinical Studies - Evolving Strategies in CRC

To date, the majority of the clinical efficacy data originate from clinical trials conducted in heavily pre-treated, chemorefractory metastatic CRC patients. Only a few studies investigated efficacy of anti EGFR mAb in first-line treatment (Bokemeyer et al., 2009; Van Cutsem et al., 2011) resulting in improved response rates in chemonaive patients.

Furthermore the concept of simply moving targeted drugs and biomarkers approved in metastatic patients to an adjuvant or early setting has failed, which was shown for two major antibodies: cetuximab and bevacizumab. Surprisingly, cetuximab in addition to FOLFOX-6 showed no benefit over a FOLFOX-6 regimen in the adjuvant setting of colorectal cancer patients in terms of disease-free survival (DSF) and response rates (Alberts et al., 2012). The lack of benefit was independent of the mutation status in the KRAS gene. Similarly, two population studies CO-8 and AVANT failed to show benefit from bevacizumab in CRC patients of stages II and III (Allegra et al., 2011; de Gramont, 2011). In fact, in the AVANT trial the outcomes of the patients were slightly worse with bevacizumab in comparison to the chemotherapy alone. These findings stand in contrast to the positive effects of mAb observed in the subset of metastatic colon cancer patients.

It is not surprising though, that such negative results influenced considerations on a new preclinical and clinical trial strategy as it has stayed largely unchanged during the last 30 years (Doroshow and Parchment, 2008). Clinical trials are focused on large populations of patients with poorly characterized disease. They take years to complete, achieving little impact on the progress in the treatment strategies.

Lack of rational clinical trial design affects not only response rates to the drugs that are already approved, but raises as well concerns about current anti-cancer drug development process (de Bono and Ashworth, 2010). Only 5-10% of potential therapeutic molecules, which showes promising preclinical data, progress to phase III clinical development. Even fewer targeted agents are approved. That suggests a systematic problem already in the preclinical phase, which determines whether the experimental agent will, at all, undergo subsequent steps of drug development. One reason for failure is the expectation that these new targeted drugs are effective in a large fraction of patients recruited to the trials. Recent findings however, indicate that many of the targeted drugs show limited benefits, often only in small subgroups of patients. This highlights the need to incorporate pharmacogenomic markers of response to drug treatment into anti-cancer development, and the discovery and validation of predictive biomarkers along with the discovery of novel therapies. Such studies should be conducted in tumours that are well characterized concerning their genetic, epigenetic, and phenotypic background. Nevertheless, before clinical trials can be initiated to test a novel compound in actual patients, researchers need to assess the drug's efficacy and safety in the preclinical study. A proper preclinical approach can facilitate the approval in the further clinical phases. In vivo as well as in vitro systems served so far in cancer modeling for preclinical testing.

1.6.2 Mouse Models of Cancer

Conventional transgenic or knockout mouse models are genetically engineered animals in which an existing gene has been inactivated or replaced, or a foreign DNA has been integrated into the murine genome. In cancer models they are generated to carry cloned oncogenes or to lack tumour suppressor genes. They cannot, however, mimic sporadic cancers, because introduced mutations are present in all cells of the body.

Moreover, certain patterns of alterations characteristic for a particular cancer cannot be introduced in the germ line due to their lethal effect. Finally, it is difficult to design a mouse predisposed to particular cancer as most mutations are not cancer specific and there is no way to predict the effect of mutations introduced in the germline to the cancer development in this mouse. The second generation of the transgenic mice – conditional models was developed to circumvent embryonic lethality and unwanted tumourigenesis. They carry regulatable oncogenes and provide an opportunity to induce somatic mutations in tissue-specific and time-controlled fashion (Jonkers and Berns, 2002). Although conditional mouse models offer an important advantage over conventional models, the complexity and diversity of the molecular alterations lead to challenges in engineering of such models to mimic the formation of sporadic cancer.

1.6.3 Xenograft Mouse Models Established from Human Cancer Cell Lines

Another approach to apply mouse models in cancer drug research was the use of immunodeficient mice that carry actual human tumours. Few research groups explored such xenograft models derived of human cancer cell lines. Some of them investigated pharmacokinetics and efficacy of cetuximab alone or in combination with chemotherapy in series of xenograft models established from various human colorectal cell lines (Balin-Gauthier et al., 2006b; Luo et al., 2005a; Prewett et al., 2007b). Although these xenografts models use human cell lines, the method still has numerous limitations.

Cell lines are adapted to grow in laboratory, often for decades, which can affect their behavior. Although it is believed that, on average, cell lines preserve parental genetics and histology, significant mutations needed for their immortalization, may as well alter their properties and biology. After several passages the cells develop a capability of an unlimited division as long as they are supplied with nutrients. Such immortalized cell cultures are generated due to the cell selection. They may involve such features as reduced cell size and higher cloning efficiency. They may also contain variable chromosome number or altered mutation pattern. Therefore culturing may lead to a low biological relevance with the organism from which the cell line origins (Sharma et al., 2010; Caponigro and Sellers 2011). As well the limited number of available cancer cell

lines indicates that they cannot well reflect heterogeneity and individuality of cancer patients.

1.6.4 Xenograft Models of Patient-derived Tumours

Disadvantages related to the use of cell lines in preclinical trials can be eliminated by transplantation of cancer tissues derived directly from the patients. Human tumour xenografts, originated directly from cancer patients, represent the heterogeneity and individuality of each malignoma. In contrary to the cell-lines-derived models, patient-derived xenografts retain the properties of the donor tissue in regard to the histology and genotype. As well tumour micro-environment and the growth properties are similar to the original patients' tumour tissue. Well characterized models are of vital importance for preclinical research in the development of novel anti-cancer drugs. As the emphasis in cancer research has shifted from the "one-fits-all" attitude to personalized medicine, such patient-derived xenograft models can be use to individualize the approaches to cancer treatment. They allow testing various cancer drugs on the individual patient tumour and selecting the most effective therapy. Furthermore, xenograft models provide sufficient tissue material for molecular studies of biomarkers that are predictive for response/resistance to therapy.

Such panels of patient-derived models were so far created for cancer types as lung cancer, breast cancer and for metastatic colon cancer (Fichtner et al., 2008; Bertotti et al., 2011; Reyal et al. 2012).

1.7. **OBJECTIVES OF THE STUDY**

- To perform molecular characterization of a panel of 133 xenograft models derived of chemonaive primary tumours of colorectal cancer patients across four UICC stages in the context of their entity with the original primary tumour

- To analyze response/resistance towards approved targeted monoclonal antibodies including cetuximab and bevacizumab as well as the standard chemotherapy agent - oxaliplatin in a subset of 67 xenograft models

- To validate the best sequential combination of the known predictive biomarkers, such as mutations in KRAS or BRAF for anti-EGFR mAb, in relation with the response to the administered therapies

- To identify novel predictive biomarkers on the global expression level of mRNA and miRNA or mutation patterns of response/resistance and test their accuracy in comparison to the established markers

Chapter 2

Patients, Materials, and Methods

2. 1 EQUIPMENTS AND MATERIALS

Instuments & Equipment	Supplier
ABI Fast 7500 instrument	ABI
Affymetrix GeneChip Fluidics Station 450	Affymetrix
Affymetrix GeneChip Scanner 3000	Affymetrix
Agilent 2100 Bioanalyzer	Agilent Technologies
Analog Vortex Mixer	VWR International GmbH
Bionanalyzer Chip-Priming Station	Agilent Technologies
Canon PowerShot G5	Canon
Centrifuge 5804R	Eppendorf 5804R
Cryostat Leica CM3050 S	Leica
Drying closet	Memmert
Electrophoresis Power Supply Source TM 300V	VWR International GmbH
Gel Photo Chamber	Biometra
GeneChip Hybridization Oven 640	Affymetrix
Laboratory Scale	Sartorius analytic
Microcentrifuge (Conventional/Butterfly rotor)	Roth
Microscope	Leica BM E
PCR Thermal Cyclers Uno	VWR International GmbH
pH-meter	Hanna Instruments
QIACube robot	Qiagen
Sliding microtome, Shandon Finesse	Thermo Fisher Scientific
Spectrophotometer, Nanodrop	GE Healthcare
Spectrophotometer, Nanovue	GE Healthcare
SubCell GT Electrophoresis system	Biorad
Thermocell Mixing Block	BIOER
Vaccum Pump	Leroy-Somer
Water Bath	Störk-Tronic

Kits	Supplier
Agilent RNA Nano 6000 Kit	Agilent Technologies
AllPrep DNA/RNA Mini Kit	Qiagen
FlashTag Biotin HSR RNA Labeling	Genisphere
High Capacity cDNA Reverse Transcription Kit	ABI
Invisorb Spin Blood Maxi Kit	Invitek
Invisorb Spin Tissue Mini Kit	Invitek
Invisorb Spin Tissue Mini Kit	Invitek
MessageAmp™ Premier RNA Amplification Kit	Ambion
miRNeasy FFPE Mini Kit	Qiagen
miRNeasy Mini Kit	Qiagen
TaqMan Reverse Transcription Reagents	ABI
TwinSpin Cell Mini Kit	Invitek

Consumables	Supplier
0,2 µm Cellulose Membrane Filter	Roth
Adhesive Film	Sarstedt
Eppendorf Pipettes 10; 100: 200; 1000µl	Eppendorf
Folded Filters 240mm	Roth
Solidofix, Freezing Spray	Roth
Hypodermic Needles	Braun
Laboratory Glass- and Plasticware	Roth
MicroAmp Fast Optical 96-Well Reaction Plate	ABI
MicroAmp Optical Adhesive Film	ABI
Microscope Glas Slides 76x26 mm	Roth
Microtube Tough-Spots	Diversified Biotech
Multichannel Transferpipettes Brand 0.5-10; 2-20; 20-200uL	Brand
Multiply Fast PCR Plates	Sarstedt
Pasteur Pipettes	Roth
Reaction Tubes 0,2; 0,5; 1,5; 2 ml	Sarstedt
SafeLock 2 ml tubes	Eppendorf
Sterile Disposable Scalpels	Braun
Tips with Filter 2,5; 10; 100, 1000 µl Biosphere	Sarstedt
Tissue Freezing Medium	Jung
Tissue Wipers Precision Wipes	Kimberly Clark Science

Conventional PCR/Electrophoresis	**Supplier**
Broad Range Agarose	Roth
50 bp DNA Ladder	Roth
6 x Gel Loading Solution	Sigma
1 kb DNA Ladder	Sigma
Ethidium Bromide	Roth
Taq Polymerase	Invitek
PCR Buffer	Invitek
$MgCl_2$	Invitek
dNTPs 100mM stock solution	Invitrogen

Chemicals and others	**Supplier**
100% Acetic acid	Roth
Anti-streptavidin antibody (goat), biotinylated	Vector Laboratories
20x Eucaryotic Hybidization Controls (bioB, bioC, bioD, cre)	Affymetrix
Acid hematoxylin solution Mayer	Roth
Control B2- Oligonucleotides	Affymetrix
Biotinylated Anti-Streptavidin	linaris
Boric Acid	Roth
Bovine Serum Albumin (BSA)	Roth
Dimethyl sulfoxide (DMSO)	Sigma
EDTA	Roth
Eosin G	Roth
Formamide	Roth
Goat IgG Protein	Dunnlab
Herring Sperm DNA Solution	Invitrogen
MES hydrate	Sigma
MES Sodium Salt	Sigma
NaCl	Roth
NaH_2PO_4	Roth
NaOH	Roth
Paraformaldehyde	Roth
Phosphate buffered saline, tablets	Gibco
RNaseZap Wipes	Invitrogen
Rotisol	Roth
SAPE (streptavidin, R-phycoerythrin conjugate)	Invitrogen
Sodium Dodecyl Sulfate (SDS)	Roth
Tris Base	Roth
Tween-20	Roth
Water	Gibco
Xylol	Roth

Databases and Internet References	Web Adress
Cancer Statistics - Americam Society of Cancer	http://www.cancer.org/research/cancerfactsfigures/index
Cancer Statistics -GLOBOCAN	http://globocan.iarc.fr/
Clinical Trials - National Cancer Institute	http://www.cancer.gov/clinicaltrials
Clinical Trials - National Institutes of Health	http://clinicaltrials.gov/
COSMIC Database	http://cancer.sanger.ac.uk/cosmic/
DAVID	http://david.abcc.ncifcrf.gov/
Ensembl	http://www.ensembl.org/index.html
IARC TP53 Database	http://p53.iarc.fr/
miR Disease	http://watson.compbio.iupui.edu:8080/miR2Disease/
miRBase	http://www.mirbase.org/
mycancer genome	http://www.mycancergenome.org/
NCBI	http://www.ncbi.nlm.nih.gov/
Sequence converter	http://www.fr33.net/seqedit.php
Tumorzentrum Berlin-Brandenburg	http://www.tumorzentrum-brandenburg.de
UCSC Genome Browser	http://genome.ucsc.edu/

Software	Supplier
2100 expert Software	Agilent Technologies
7500 Software v2.0.1	ABI
Chromas Lite 2.1	Technelysium Pty Ltd
Finch TV	Geospiza
GeneChip Command Console Software (AGCC)	Affymetrix
GraphPad Prism 5 software for Windows	GraphPad Software Inc
Microsoft Office Package	Microsoft
miRNA QC tool (Affymetrix)	Affymetrix

2.2 PATIENT POPULATION

2.2.1 MSKK and RVS - Trials in CRC

Sample source - MSKK Study

Since 2005 the MSKK study recruited almost 7.000 colorectal cancer patients who underwent surgery and received a standard treatment according to the German S3 guidelines. This multi-center, prospective clinical trial enrolled colorectal cancer patients of all four UICC stages from 37 primary care and university hospitals in Germany. From most of the patients fresh-frozen and formalin-fixed paraffin-embedded (FFPE) tumour specimens as well as several blood samples were collected using a standardized procedure. Mutation analysis of KRAS, BRAF and PIK3CA was conducted in a cohort of 788 patient samples originating from the MSKK study. The 788 patients underwent surgical resection in sixteen hospitals between 2005 and 2008.

Sample source - RVS Study

A subset of frozen tissue samples assessed in mutation analysis was collected in a framework of another clinical trial entitled Retrospective Validation Study (RVS). This study enrolled app. 1000 CRC patients of which 172 patient samples were used for mutations analysis. These 172 patients underwent surgical resection between 1995 and 2005 in three hospitals.

Ethical Principles

All samples were collected according to the ethical requirements and regulations. In parallel, comprehensive individual clinical data were collected by clinical research associates using a FDA approved electronic data capturing software. All patients have given their written informed consent to participate in the clinical studies. Basic clinical and pathological characteristics of the patients are shown in the Tab.3.6. Beside the fact that one clinical study was prospective and the other retrospective, a mixture of the two studies seems to be a random representation of the patients' population according to the basic clinicopathological characteristics and distribution of analyzed mutations.

2.2.2 Exclusion Criteria and the Final Samples Selection

Failure of the molecular analysis

Mutation analysis of KRAS, BRAF and PIK3CA was performed in 960 tumour samples originated from the MSKK and the RVS studies. KRAS mutation status could not be assigned to 20 samples, BRAF mutation status to 10 samples and PIK3CA analysis failed in 22 samples. When an assay did not succeed in one of the analyzed hotspots, such sample was omitted in further analyses. In this manner 47 samples were excluded. Forty-one (87%) out of the 47 failing DNA samples were isolated from the FFPE tissues and only six from the snap-frozen tumour specimens.

Exclusion of the duplicates

Altogether, 913 samples remained for further analysis after exclusion of those, in which mutation analysis did not succeed. It turned out that in 34 cases more than one tissue sample was analyzed per patient. In 22 out of the 34 cases one sample derived from FFPE and another from snap-frozen tissue. Analysis of the 22 matched sample pairs revealed that in six cases mutation results did not match. Five of the 22 cases were patients with synchronous disease (two different tumours). Analysis of another five cases with synchronous cancer where two samples were available from each patient revealed, however, no differences in mutation status in both tumours (three were wild-type in analyzed hotspots, two carried mutations in KRAS: G12D and G13D). Altogether there were ten cases of synchronous cancer with two samples of each patient analyzed. In five cases both tumours matched the genotype, while in other five, they did not match. For statistical analyses only the result of the more advanced tumour was taken under consideration. Duplicates were excluded. In one case, a discrepancy was found in the mutation status of a patient with no diagnosis of synchronous cancer. According to the analysis of fresh frozen tumour this patient was wild-type in analyzed hotspots, while DNA isolated from FFPE showed mutations in KRAS (G12D) and PIK3CA (E545K). A reason for the discrepancy between frozen and FFPE samples of the same patient might be the fact that tissue samples were taken from different regions of the tumour (tumour center, invasion front etc.). Of the two, the sample originating from the tissue part that was closer to the tumour centre was included in the analysis.

Pathological and clinical exclusions

Another six cases were excluded due to the violation of the inclusion criteria of the study. Pathological evaluation revealed that additional seven samples represent relapses (5),

metastasis (1), or biopsy (1) instead of primary tumours. Finally, in two patients, FAP was diagnosed resulting in exclusion of these samples from further analysis. Thirty patient samples which were analyzed for mutation turned out to originate from patients diagnosed, apart from colorectal cancer, with another malignancy. These were not excluded from mutation analysis. Also the 42 cases in which tumour samples originated from the patients who received neoadjuvant treatment prior to surgery were not excluded.

All tissue samples were analysed for their tumour content, but also for the content of other cell types by board certified internal pathologists. This semiquantitative data was recorded for each tumour sample in a respective database. In 19 samples tumour content in the tissue as evaluated by pathologist was lower than 10%. Most of these samples were resected after neoadjuvant treatment or from mucinous tumours, which are characterized by low cellularity. Ten mutations were found in eight out of these samples, out of which seven mutations were identified in samples with a tumour content below 5%. Due to the reasonable mutation rate and in order not to select against mucinous tumours, the samples with low tumour content were not excluded from further analyses.

2.3 ESTABLISHMENT OF THE XENOGRAFTS

NOD/SCID mice (Taconic)

RPMI-1640 medium containing gentamicin

Two hundred and thirty-nine fresh tumour samples originating from the MSKK study were used to establish a panel of patient-derived xenograft models. Shortly after surgery original tumour pieces were shipped in RPMI-1640 medium containing gentamicin to the collaborating company - Experimentelle Pharmakologie & Onkologie Berlin-Buch GmbH (EPO). There, samples were cut into pieces of 3 to 4 mm and transplanted within 30 min. to 3 - 6 immunodeficient NOD/SCID mice (Taconic). The gender of recipient mice was chosen according to the gender of donor patient. In this manner 239 fresh human tumour samples were transplanted into NOD/SCID mice. All transplanted samples were collected during two years by a collaborating network of four primary care hospitals. Tumours were further passaged until stably growing tumour xenografts developed. Hundred and forty-nine out of 239 samples (62%) were successfully engrafted. A quality control of the engrafted tumours led to the exclusion of 16 models. Among excluded engrafted tissues were metastases originated from other primary tumours and adenomas. One of the xenografts was excluded due to withdrawal of informed consent by the respective patient. Tumour specimens from patients who had received neoadjuvant

treatment were excluded, as one of the goals of the study was to test the efficacy of anti-cancer compounds in the chemonaive population. Finally, 133 high-quality xenografts passed quality control and fulfilled criteria of the study. In this way a panel of 133 stably passagable, patient-derived colorectal cancer xenografts could be established as permanent tumour models. All animal experiments were done in accordance with the United Kingdom Coordinating Committee on Cancer Research regulations for the Welfare of Animals and of the German Animal Protection Law and approved by the local responsible authorities (Landesamt für Gesundheit und Soziales).

2.4 TREATMENT EXPERIMENT

NMRI: nu/nu mice (Charles River)

Cetuximab (erbitux; Merck), qd 7x2, 50 mg/kg/d, i.v.

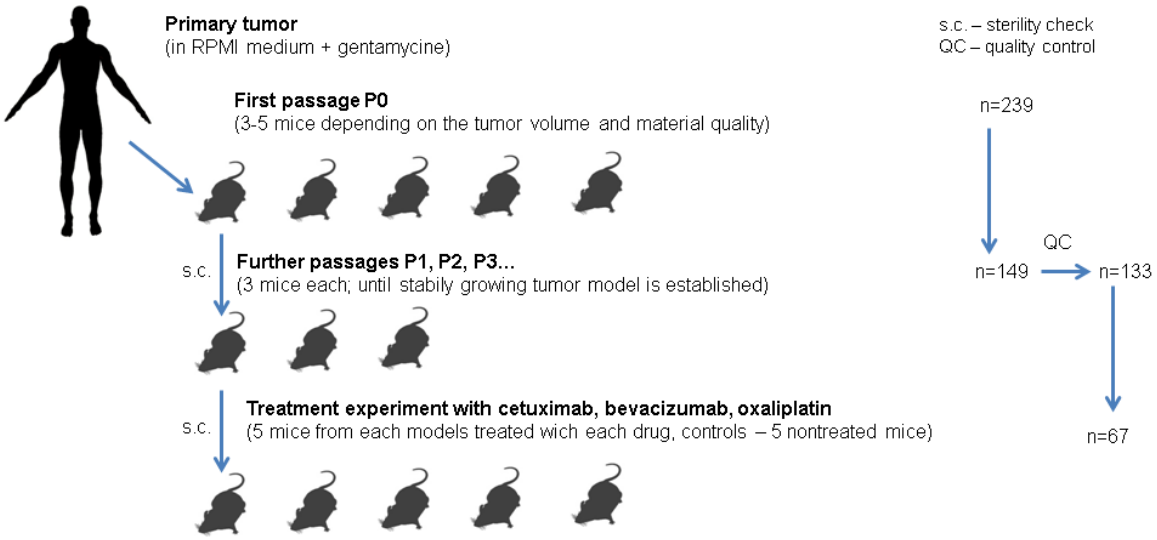
Bevacizumab (avastin; Genentech Inc., South San Francisco, CA, USA), qd 4, 5 mg/kg/d, i.p

Oxaliplatin (eloxatin, Sanofi-Avensis), 5 mg/kg/d, qd 1-5, i.p.

Sixty-seven of the 133 established xenograft models were used in therapy experiments (Fig.2.1). Three well known and approved drugs were tested as single agents: oxaliplatin which forms the backbone of the FOLFOX regimen, and two targeted anti-EGFR antibodies - cetuximab and bevacizumab. Mice were observed daily for tumour growth. At a size of app. 1 cm^3, 67 tumours were removed and passaged to naive, male NMRI: nu/nu mice (Charles River). Each tumour was transplanted subcutaneously to five mice. Treatment was initiated at palpable tumour volume of app. 50-100 mm^3. For every out of 67 human-derived xenograft models, five mice were tested with each drug and five additional mice were used as controls. The following drugs and treatment schedules were used: cetuximab (Erbitux; Merck) qd 7x2, 50 mg/kg/d, i.v.; bevacizumab (Avastin; Genentech Inc., South San Francisco, CA, USA) qd 4, 5 mg/kg/d, i.p.; oxaliplatin (Eloxatin, Sanofi-Avensis), 5 mg/kg/d, qd 1-5, i.p. Doses and schedules were chosen according to the previous experience in animal experiments and represent the maximum tolerated dose of oxaliplatin and the efficient doses of monoclonal antibodies cetuximab and bevacizumab (see Tab.3.7). The injection volume was 0.2 mL/20 g body weight.

Tumour size was measured twice a week with a caliper-like instrument in two dimensions. Individual tumour volumes (V) were calculated by the formula: V = (length + [width] ^{2}) / 2 and related to the values at the first day of treatment (relative tumour volume). Median treated to control (T/C) value of relative tumour volume was used for the evaluation of each treatment modality (Fichtner et al., 2008).

Fig.2.1. *Workflow scheme of transplantation of the 239 CRC patients tumour tissues, engraftment of 149, and treatment of 67 of the engrafted models with cetuximab, bevacizumab and oxaliplatin; s.c. - sterility check; QC - quality control*



2.5 TISSUE SECTIONS AND PATHOLOGICAL EVALUATION

4% solution of paraformaldehyde in PBS

Acid hematoxylin solution Mayer; eosin G

FFPE samples - preparation of the sections

FFPE blocks were cut using a sliding microtome (Shandon Finesse, Thermo Fisher Scientific). Prior to cutting, paraffin-blocks were cooled by a freezing spray (Solidofix). First 4 µm primary sections were prepared and placed in the water bath. Afterwards, they were collected with a suitable glas slide and left for two hours to dry. Next, sections were stained with haematoxylin and eosin (H&E). H&E staining protocol used for the FFPE slides is shown in Tab.2.1.A. Pathological evaluation of the primary sections included tumour classification according to their morphological and histological features. Content of tumour cell and other cell types was estimated in a semiquantitative manner. If possible, the areas to be macrodissected were marked. Macrodissection was assessed to enrich the content of tumour cells in the sample and to minimize the content of other tissues (example - Fig.2.2). Marked tumour region was excised with a needle and thereafter only excised fragments were collected into a tube. According to the pathologists' guidelines, between 2 and 10 sections of 20 µm thick FFPE sections were cut for DNA and RNA isolation. Before and after sectioning, additional 4 µm-thick sections

were cut and stained with H&E in order to evaluate whether the tissue structure had changed depending on the thickness of the FFPE block.

Snap-frozen samples - preparation of the sections

Sections of the snap-frozen tissues were prepared using the Leica CM3050 S cryostat. Tissues were embedded using a freezing medium (Jung) and transferred to a cryotome (-20°C). After app. 5 min., tumour tissues were immobilized in the medium and their temperature equilibrated to the temperature of the cryostat. Next, sections of the desired thickness were cut. Tissue sections were placed on the glass slides and transfered within 30 min. to the 4% paraformaldehyde solution for app. 5 min. Afterwards snap-frozen sections were stained with H&E following the steps listed in Tab.2.1.B. Macrodissection of the snap-frozen specimens was assessed with a scalpel. Between 10 and 100 of 4 µm-thick sections were used for DNA and RNA isolation according to the pathologists' instructions. Except for the mentioned differences, snap-frozen sections were prepared and evaluated by pathologists analogously to FFPE sections.

Tab.2.1. *Hematoxylin & Eosin Staining Protocol*

A. *For the FFPE slides*

Step	Reagent	Duration
1	Xylol	5 min.
2	Xylol	5 min.
3	Xylol	5 min.
4	100% Ethanol	5 min.
5	96% Ethanol	5 min.
6	90% Ethanol	5 min.
7	80% Ethanol	5 min.
8	70% Ethanol	5 min.
9	Distilled Water	5 min.
10	Haematoxylin (Meyer)	3 min.
11	Tap Water	Wash
11	Tap Water	15 min.
12	1% Eosin	3 s
13	Distilled Water	Wash
14	70% Ethanol	Wash
15	80% Ethanol	Wash
16	90% Ethanol	Wash
17	96% Ethanol	Wash
18	100% Ethanol	Wash
19	Xylol	3min.
20	Cover the slide	-

B. *For the snap-frozen slides*

Step	Reagent	Duration
1	100% Ethanol	5 min.
2	96% Ethanol	5 min.
3	90% Ethanol	5 min.
4	80% Ethanol	5 min.
5	70% Ethanol	5 min.
6	Distilled Water	5 min.
7	Haematoxylin (Meyer)	4 min.
8	Tap Water	Wash
9	Tap Water	15 min.
10	1% Eosin	4 s
11	Distilled Water	Wash
11	70% Ethanol	Wash
12	80% Ethanol	Wash
13	90% Ethanol	Wash
14	96% Ethanol	Wash
15	100% Ethanol	Wash
16	Xylol	3min.
17	Cover the slide	-

A. B. C.

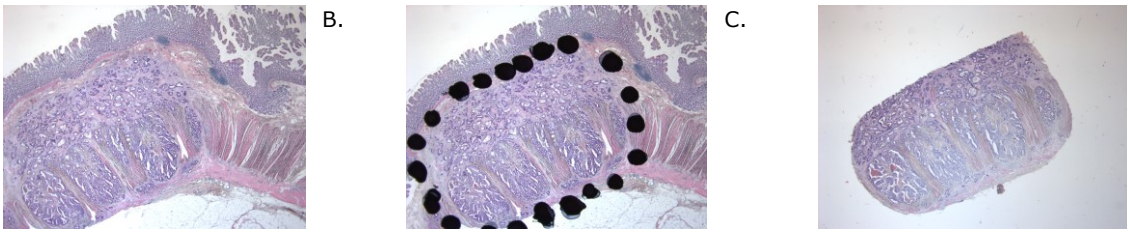


Fig.2.2. *Example of tumour cell enrichment by macrodissection: [5X] A) Tissue section of colonic adenocarcinoma, stained with H&E; epithelial tumour: 20%, smooth muscle: 30%; stroma: 20%; normal epithelium: 30% B) Tissue section with areas marked for macrodissection C) Tissue section after macrodissection; epithelial tumour: 60%, smooth muscle: 20%; stroma 20% (Photos: Dr. I. Klaman)*

2.6 MOLECULAR ANALYSES

2.6.1 DNA and RNA Extraction

Paraffin disposal and DNA isolation from FFPE samples

Verdau-Puffer (30mM Tris, 10mM EDTA, 1% SDS, pH 8,0); proteinase K; 5M NaCl; 70% ethanol

Genomic DNA from FFPE tissue was isolated using Invisorb Spin Tissue Mini Kit (Invitek) preceded by paraffin disposal. Prior to the isolation, FFPE sections were overnight incubated on the thermoshaker at 55°C with an addition of Proteinase K. After overnight digestion, a 30 min. long centrifugation at 4°C was performed. As a result debris moved to the bottom of the tube. Paraffin on the other hand formed a ring in the upper part of

the tube, allowing its separation from the supernatant. 5M NaCl was added to the supernatant to the final concentration of 100 µM and then DNA was precipitated with absolute ethanol. The precipitate was washed with 70% ethanol, dried, and finally dissolved in the lysis buffer G. Thereafter, the isolation was conducted according to the manufacturer's protocol.

DNA/RNA isolation from snap-frozen samples

Genomic DNA from snap-frozen primary tumour tissue was isolated along with the total RNA using Invisorb TwinSpin Cell Mini Kit (Invitek) in a spin-filter format. From the 133 xenograft snap-frozen samples DNA and RNA were simultaneously extracted with AllPrep DNA/RNA Mini Kit (Qiagen) using an automated protocol on the QIACube robot (Qiagen) according to the manufacturer's instructions.

DNA isolation from blood and cell lines- positive and negative controls

gDNA that served as a negative control in the mutation analyses was extracted using Invisorb Spin Blood Maxi Kit (Invitek) from the blood of healthy patients that underwent colonoscopy. DNA that was used as a positive control was extracted from the snap-frozen cell lines. It was isolated using Invisorb Spin Tissue Mini Kit (Invitek) according to the manufacturer's protocol.

Total RNA isolation from FFPE samples

RNA from the FFPE tissues of the CRC primary tumours was extracted by automated protocol (QIACube) using miRNeasy Mini Kit (Qiagen, Hilden). Total RNA from the control tumours of the 67 models used in the treatment experiment was isolated using the miRNeasy FFPE Mini Kit (Qiagen, Hilden).

Quality control in nucleic acid isolation - DNA and RNA concentration measurements

DNA and RNA concentrations (ng/µl) were measured using UV spectrophotometer (Nanovue, GE Healthcare/Nanodrop, Thermo Scientific). One or two µl of eluted DNA/RNA were assessed for the measurement with Nanovue and Nanodrop respectively.

Quality control in nucleic acid isolation - gel electrophoresis
TAE buffer (4 mM TRIS, 1.2 mM sodium acetate, 4 nM EDTA adjusted with acetic acid to pH 8)
6 x loading buffer (bromphenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v), sucrose (40% w/v))

Quality of isolated DNA was assessed in 1% agarose gel electrophoresis. Agarose gel was prepared using 130 ml of TEA buffer, 1.3 mg of broad-range agarose and 4.5 µl of 1%

ethidium bromide solution. After placing the comb/combs, app. 30 min. was needed for polymeryzation of the gel. During polymeryzation 8-10 µl of isolated DNA was combined with 2 µl of 6x concentrated loading buffer. Next, 1 kb DNA ladder (NE Biolabs) was loaded on the gel as a control, followed by loading of the isolated samples. Electrophoretic separation was performed at a voltage of 75 - 100 V for 1 to 1.5 h. The evaluation of DNA quality was made by determination of the intensity and degree of DNA fragmentation allowed by visualization of intercalated ethidium bromide under a UV lamp.

Quality control in nucleic acid isolation - RNA integrity

The integrity of RNA was measured on the Agilent 2100 bioanalyzer (Agilent Technologies). Prior to the analysis, all the RNA Nano 6000 reagents were placed for 30 min. at room temperature. Gel matrix was prepared by filtering through the supplied column (centrifugation: 10 min., 4000 rpm) and adding 1 µl of dye to the 65 µl of gel. After placing a RNA Nano 6000 chip in the priming station, 9 µl of gel-dye mix was pippetted into a G well (black). The priming station was closed and the plunger was released after 30 s. Next, the gel-dye mix was pipetted into remaing G wells (grey). Five µl of Nano Marker, 2 µl of denatured ladder and 2 µl of analyzed samples were pipetted into the chip. The samples were diluted, if the measured concentration was higher than 200ng/µl. The chip was vortexed for 1 min. at 240 rpm and inserted into bioanalyzer. A RIN (RNA integrity number) greater than 4 was required to classify the quality of the RNA sample as sufficient for further analyses.

2.6.2 Mutation Analysis

Mutation analysis was conducted by allele-specific real-time PCR using custom TaqMan-MGB allelic discrimination assays (Applied Biosystems). The sequences of the primers and probes are listed in the Tab.S3. Every well contained 20 ng of purified gDNA template. Samples were run in duplicates in the final volume of 10 µl with TaqMan Universal PCR Master Mix (2x) No AmpErase UNG, 40 x working stock of allele-specific assays and blocking oligonucleotides (125 nM). Amplification was run in the ABI Fast 7500 instrument. Standard TaqMan thermocycling conditions were used (10 min - 95°C, 40 cycles of 15 s at 92 °C, 1 min at 60°C) for all mutation analyses, except for the BRAF anneal/extend step, which was prolonged to 90 s. Every 96-well plate was composed of 40 patients' samples in duplicates, 4 negative controls (WT), 8 positive controls (titrations), and 4 non-template controls (NTC).

2.6.2.1 The principle of the allele-specific real-time PCR

Allele-specific real-time PCR was designed to detect DNA sequence variants that differ in only one nucleotide. A sequence of interest, investigated for the mutation status, is flanked by a pair of primers. Each assay contains two probes: one is labeled with a fluorescent dye FAM and another with a fluorescent dye VIC. FAM- labeled probe binds to the mutated sequence and VIC-labeled probe detects a wild-type sequence. The process of amplification leads to the hydrolysis of the probe by Taq's 5' to 3' exonuclease activity. A dye from the matched probe is released and the fluorescent signal is generated. Black hole quencher prevents fluorescence of the non-matching probe. Specificity of the assays is improved by the minor groove binder (MGB) that forms stable duplexes with the single-stranded DNA targets.

Titration - serial dilution of mutant allele with wild-type gDNA

Originally, allele-specific real-time end-point assays are designed to generate three types of signal. A substantial increase in FAM fluorescence indicates a homozygous mutant, a VIC signal indicates a homozygous wild-type, and finally increase in both signals indicates heterozygosity and an intermediate signal is generated by the software (Fig.2.3).

Majority of the somatic mutations in cancer is dominant and normally affect only a single allele of the gene. Homozygous mutations in cancer genomes occur seldom, in most of the cases as a result of the LOH (loss of heterozygosity) in which a germ-line mutation in one allele is followed by a subsequent somatic alteration. Therefore, in most of the cases both probes are binding and an intermediate signal is generated. Moreover, tumour tissue samples are genetically heterogeneous. They often contain, apart from tumour cells, a variety of other components such as surrounding stroma or the inflammatory cells. Commonly, due to the complicated sample architecture, an adjacent tissue cannot be removed by macropreparation. Only tumour cells carry somatic mutations, therefore an isolation of DNA from such a cell mixture leads to an increase of wild-type sequence fraction. Ultimately, an increase of the VIC signal is observed. Due to the challenging interpretation of the allele-specific real-time PCR, an analysis of serial dilutions of the mutant samples with the wild-type genomic DNA (titration) was conducted on every plate. In order to interpret the results and to decide whether the sample was mutated or not, a ratio of the FAM and VIC was analyzed along with the visual analysis of the scatter plots and in comparison with the titrations.

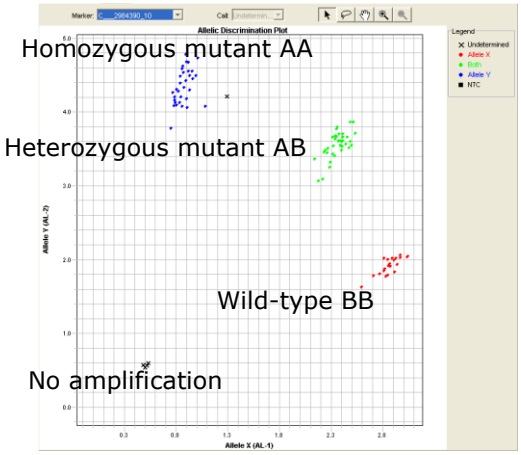


Fig.2.3. *Scatter plot illustrates endpoint determined exemplary groups of homozygous (mutant and wild-type) and heterozygous samples. Each cross represents one analyzed sample. Increase in FAM (mutant) signal shifts the samples to the upper part of the plot (blue crosses), while the fluorescent signal from VIC (wild-type) shift the samples to the right (red crosses). An intermediate signal generated from both FAM and VIC fluorescent signals (green crosses) indicated a hezerozygous sample.*

2.6.2.2 Blocking non-labeled oligonucleotides

In some of the cases, during the pilot experiments, more than one mutation was detected in the same sample, particularly in codon 12 of the KRAS gene. It was caused by the fact that six assays, which were designed to detect different mutations in KRAS codon 12, differ in one nucleotide only (Fig.2.4). Such similarity led to the binding in the fraction of the imperfectly matched probes and to generation of the unspecific signal. This cross-reactivity resulted in the challenging interpretation of the preliminary results. In order to prevent nonspecific binding, unlabeled oligonucleotides were added to the reactions. These unlabeled oligonucleotides were competitive with the unmatched probes and blocked their binding position. They prevented generation of nonspecific fluorescence signals that originated from the unspecific mutations in codon 12 of the KRAS gene. The combinations of the probes and blocking oligonucleotides are shown in table 2.2.

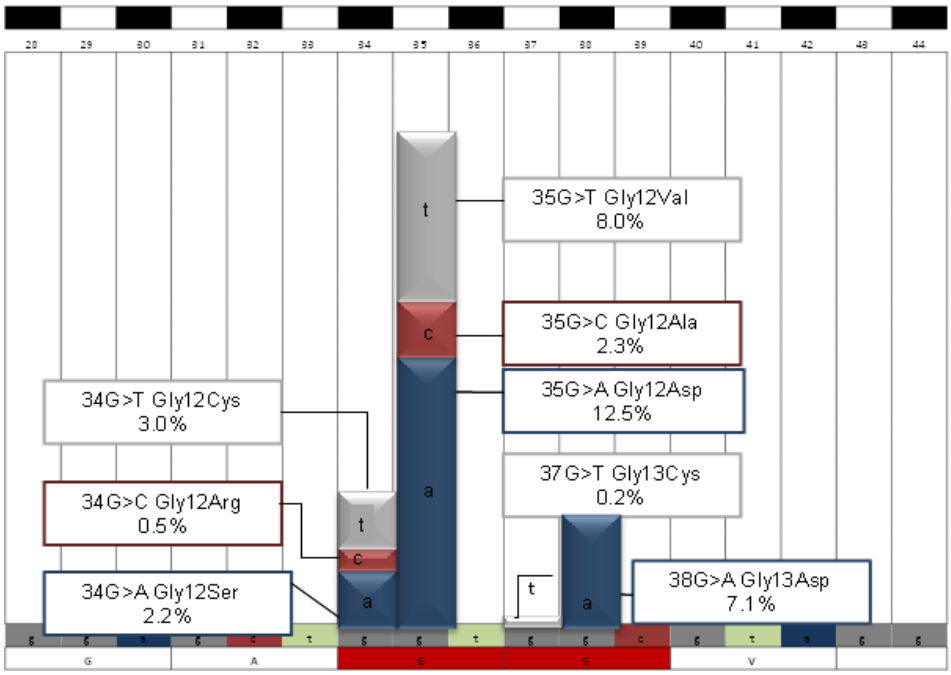


Fig.2.4. *Distribution of mutations in codon 12 and 13 in the KRAS gene and their frequency in the CRC population (source: COSMIC database - modified)*

Tab.2.2 *Combinations of the probes and blocking oligonucleotides*

Assay ID	Target Mutation	Blocker ID	Blocker sequence	Blocker(s)used in the reaction
KRAS_ex2-121a	34 G>A	Blocker_121a	TAGTTGGAGCTAGTGGCGTAG	Blocker_121t
KRAS_ex2-121t	34 G>T	Blocker_121t	TAGTTGGAGCTTGTGGCGTAG	Blocker_121a
KRAS_ex2-121c	34 G>C	-	-	Blocker_121a; 121t
KRAS_ex2-122a	35 G>A	Blocker_122a	TAGTTGGAGCTGATGGCGTAG	Blocker_122t; 122c
KRAS_ex2-122t	35 G>T	Blocker_122t	TAGTTGGAGCTGTTGGCGTAG	Blocker_122a, ; 122c
KRAS_ex2-122c	35 G>C	Blocker_122c	TAGTTGGAGCTGCTGGCGTAG	Blocker_122a; 122t

2.6.3 Direct Sanger Sequencing

2. 6. 3. 1 Direct Sanger sequencing of KRAS codon 12 and 13

Sanger dideoxy terminator sequencing was applied to verify the results obtained by the real-time PCR. KRAS status in codon 12 and 13 was analyzed by conventional sequencing of 304 samples for which allele-specific real-time PCR results were available. The primers used for amplification and sequencing were described previously (Finberg et al., 2007; Loupakis et al., 2009). Primers have the following sequences: forward - GGTGGAGTATTTGATAGTGTATTAACC and reverse - TCATGAAAATGGTCAGAGAAACC.

For each PCR reaction a master mix containing 0,2uM primers, 1,5mM $MgCl_2$, 250uM dNTPs, 1 x reaction buffer and 0.5U/uL Taq Polymerase was combined with DNA sample. The thermocycling conditions are described in Tab.2.3. The amplified product has a size of 283 bp. Traditional Sanger sequencing of the fragments was performed by LGC Genomics GmbH.

Tab.2.3 *Components (A) and conditions (B) of PCR reaction and direct sequencing of KRAS codon 12 and 13*

PCR Program	Temp. [°C]	Time	
Denaturation	94	2min	
Denaturation in cycle	94	30 s	
Annealing	61	30 s	X 35 cycles
Elongation	72	30 s	
Final elongation	72	10 min	

2.6.3.2 Direct Sanger sequencing of KRAS codon 61

Twenty-nine out of the 67 models used in the treatment experiment were not mutated in KRAS and BRAF according to the allele-specific real-time PCR analysis. In these samples mutation status in KRAS codon 61 was assessed by Sanger sequencing as reported earlier (Loupakis et al., 2009). The fragment of 155 bp was generated by primers of the

following sequences: forward - CCAGACTGTGTTTCTCCCTT; and reverse - CACAAAGAAAGCCCTCCCCA. The concentrations of the the components used in each reaction and the thermal cycling conditions are given in Tab.2.4. The same primer pair was used for sequencing of the amplified fragments. Sequencing of the fragments was performed by LGC Genomics GmbH.

Tab.2.4 *Components (A) and conditions (B) of PCR reaction and direct sequencing of codon 61 of the KRAS gene*

A)

Component	Conc.	Final conc.
Reaction buffer	10x	1x
MgCl2	25mM	1,5mM
dNTPs	12,5 mM each	250uM
Primer F	5pmole/uL	0,2uM
Primer R	5pmole/uL	0,2uM
H2O	-	-
Invi Taq	5U/uL	0.5U/uL
Template	20ng/uL	2ng/uL

B)

PCR Program	Temp. [°C]	Time	
Denaturation	95	10 min	
Denaturation in cycle	95	30 s	
Annealing	60	30 s	X 35 cycles
Elongation	72	30 s	
Final elongation	72	10 min	

2.6.4 Gene Expression Analysis of AREG and EREG by Real-time PCR

RNA Reverse Transcription - snap-frozen samples

Reverse transcription on the RNA template extracted from the snap-frozen samples was performed with TaqMan Reverse Transcription Reagents (Applied Biosystems). Reverse transcription was performed in 10 µl reaction volume. For the synthesis of cDNA 10X RT Buffer, 25 mM $MgCl_2$, dNTPs Mixture, Random Hexamers, RNase Inhibitor, and MultiScribe Reverse Transcriptase were combined with 500 ng of total RNA. The thermo-cycling conditions of the reverse transcription are described in Tab.2.5.

Tab.2.5 *Reverse transcription PCR conditionsfor snap-frozen samples*

PCR Program	Temp. [°C]	Time
Hexamer Incubation	25	10 min.
Reverse Transcription	48	60 min.
Inactivation	95	5 min.

RNA Reverse Transcription - FFPE samples

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to amplify RNA extracted from FFPE specimens. Reverse transcription of the material isolated from FFPE samples was performed in 20 µl with a starting amount of 2000 ng. Starting amount of total RNA isolated from FFPE samples was higher than in case of the snap-frozen samples because of much higher rate of RNA degradation in the FFPE tissue blocks. In order to perform reverse transcription, RNA was combined with 10X RT Buffer, 25 mM $MgCl_2$, 100 mM dNTP Mix, Random Primers, RNase Inhibitor, and MultiScribe Reverse Transcriptase and processed with the thermo-cycling conditions described in Tab.2.6.

Tab.2.6 *Thermo-cycling conditions of the reverse transcription PCR for FFPE samples*

PCR Program	Temp. [°C]	Time
Hexamer Incubation	25	10 min.
Reverse Transcription	37	120 min.
Inactivation	85	5 min.

Specific gene amplification

Material obtained in the reverse transcription reaction was diluted in proportion 1:5 and used in the specific amplification of the selected genes. Expression of AREG, EREG, PTEN, DUSP6, SLC26A3, PTPRF and LOC158960 was measured with an assays recommended by the supplier (sequences of the primers and probes are listed in Tab.S4). Analysis was performed in triplicates, in half of the volume, according to the manufacturer's instructions with a threshold set on 0.2. 20x TaqMan Gene Expression Assay, 2 x TaqMan MasterMix was combined with diluted cDNA template. Tab.2.7 shows the conditions of the amplification.

Tab.2.7 *Thermo-cycling conditions of the target-amplification (Gene Expression Assay) for FFPE samples*

PCR Program	Temp. [°C]	Time
Activation	50	2 min.
Denaturation	95	10 min.
Cycling x 40	95	15 s
	60	1 min.

Normalization of the measured expression values

In order to normalize the results, along with the measurements of the target genes, RNA levels of three housekeeping genes: GAPDH, RPLP0, and UBC were included in all analyses. All chosen probes were recommended by the manufacturer (Applied Biosystems) for the selected genes. The median of the three measurements was calculated for each of the genes. For the three housekeeping genes the mean of the medians was calculated. As the expression level indicator for every target gene (AREG, EREG, DUSP6, SLC26A3, PTPRF, and LOC158960) a difference between the median Ct value and the mean of the Ct values for three housekeeping genes (GAPDH, RPLP0, and UBC) was calculated. Pearson Correlation Coefficients (PCC) between response to cetuximab (as indicated by the T/C ratio) and log-transformed expression measurements of AREG, EREG, PTEN, DUSP6 and SLC26A3 were calculated.

2.6.5 Gene Expression Profiling (GEP) on the U133 Plus 2.0 GeneChip

1 x Array Holding Buffer (1x 100mM MES, 1M [Na+], 0,01% Tween-20)
2 x Hybridization Buffer (1x 100mM MES, 1M [Na+], 20mM EDTA, 0,01% Tween-20)
Hybridization components: BSA, Herring Sperm DNA Solution, Biotinylated Anti-Streptavidin, B2-Oligo, Dimethyl sulfoxide (DMSO); Fornmamide; 2 x 2 x Hybridization Buffer
Wash Buffer A (6x SSPE, 0.01% Tween-20)
Wash Buffer B (100mM MES, 0,1M [Na+], 0,01% Tween-20)
Staining Cocktails: Staining Buffer (100mM MES, 1M [Na+], 0.05% Tween-20); BSA; Goat IgG 10ng/µl in 150mM NaCl; SAPE; Biotinylated anti-streptavidin Ab

Reverse Transcription to Synthesize First-Strand cDNA

250 ng of total RNA was amplified and labeled using MessageAmp™ Premier RNA Amplification Kit following the manufacturer's instructions. Reverse transcription led to the synthesis of the first strand cDNA containing a T7 promoter sequence. First strand synthesis was assessed in 10 µl volume. Total RNA was mixed with the serial dilution of Poly-A RNA Control (1:500000), combined with First-Strand Buffer Mix and First-Strand Enzyme Mix and incubated for 2 hours at 42 °C.

Second-Strand cDNA Synthesis

Synthesis of the second strand converted the single-stranded cDNA into a double-stranded template for transcription. 20 µl of Second-strand Master Mix (Second-Strand Buffer Mix, Second-Strand Enzyme Mix, and Nuclease-free Water) was mixed with the single strand cDNA and incubated for 1 hour at 16 °C, followed by 10 minutes at 65 °C.

Second-strand cDNA synthesis occurs simultaneously with a degradation of the RNA by RNase H.

In Vitro Transcription to Synthesize Labeled aRNA and aRNA purification

Next, the IVT Master Mix was prepared by combining IVT Biotin Label, IVT Labeling Buffer and IVT Enzyme Mix with the double-stranded cDNA sample. The IVT reaction was incubated at 40°C for 16 hours. During In Vitro Transcription step multiple copies of biotin-modified aRNA were synthesized and amplified. Generated aRNA copies were then purified in order to to remove enzymes, salts, and unincorporated nucleotides and thus improve their stability. Purification was performed using RNA Binding Beads. Beads were combined with aRNA Binding Buffer Concentrate and the mixture was added to the aRNA sample. Next, 120 µL of 100% ethanol was added to each sample. The mixture was placed at the plate shaker for app. 2 min. Afterwards it was transferred to the magnetic stand for 5 min. in order to capture the beads and discard the supernatant. Next, the beads were washed with aRNA Wash Solution and the purified aRNA is eluted with aRNA Elution Solution.

Fragmentation and Hybridization onto GeneChip Array

aRNA yield was assessed using the Agilent bioanalyzer and RNA 6000 Nano Kit (see 2.6.1 *Quality control in nucleic acid isolation - RNA Integrity)* and 15 µg of aRNA was used for fragmentation. aRNA was mixed with 5x Array Fragmentation Buffer and nuclease-free water. The fragmentation reaction was incubated at 94 ºC for 35 minutes and afterwards it was placed on ice. Fragmented and labeled aRNA was combined with BSA, herring sperm DNA solution, biotinylated anti-streptavidin, B2-oligonucleotides, DMSO, and the hybridization buffer. The hybridization cocktail was heated to 99 °C for 5 minutes and then injected on the array. Arrays were incubated with rotation (60 rpm) for 16 h at 45°C in the oven to allow hybridization of the fragmented aRNA onto the GeneChip array.

Washing, staining and detection

Arrays were washed and stained with streptavidin-phycoerythrin in the Affymetrix Fluidics Station 450 using the EukGE WS2v4 protocol. Wash buffer A, wash buffer B, array holding buffer, staining cocktails 1 and 2 were applied during the procedure. Scanning was performed with the Affymetrix GeneChip Scanner 3000, according to the manufacturer's protocol. As a control served commercial human brain reference RNA (Ambion).

Biomarker discovery using GEP data

An Affymetrix U133 Plus 2.0 arrays approach was also used to develop novel predictive biomarkers of response to the therapies. RNA was isolated from tumour tissue of the non-treated controls. Out of 67 models that were used in the treatment experiment, RNA was available for 66 of them and pooled prior to the hybridization on the array. 18 responders and 48 non-responders were analyzed in the balanced approach. The FARMS condensation left 14.753 informative probesets. Prospective performance during balanced discovery was performed as well on the panel of corresponding primary tumours (18 responders vs. 43 non-responders). After condensation with FARMS 17.501 informative probesets were available for a discovery of predictive signature directly in the patients' tumour tissues.

Data analysis included feature selection and classification using the random forest algorithm and support vector machine (SVM) in a nested bootstrap approach. In every loop all samples are randomly split into a training set and a test set. The training set is used for signature selection. Performance of each classifier discovered in this manner is then evaluated as applied to the test set. Repeated procedure, after a large number of loops, allows revealing an average signature of best performance and high stability. Such procedure was used to develop RNA signatures discriminating between cetuximab responders and the resistant cases.

2.6.6 microRNA Expression as Measured GeneChip miRNA 2.0 Arrays

1 x Array Holding Buffer (1x 100mM MES, 1M [Na+], 0,01% Tween-20)

2 x Hybridization Buffer (1x 100mM MES, 1M [Na+], 20mM EDTA, 0,01% Tween-20)

Hybridization components: BSA, Herring Sperm DNA Solution, Biotinylated Anti-Streptavidin, B2-Oligo, Dimethyl sulfoxide (DMSO); 2 x Hybridization Buffer

Wash Buffer A (6x SSPE, 0.01% Tween-20)

Wash Buffer B (100mM MES, 0,1M [Na+], 0,01% Tween-20)

Staining Cocktails (1, 2): Staining Buffer (100mM MES, 1M [Na+], 0.05% Tween-20); BSA; SAPE (1); Goat IgG 10ng/µl in 150mM NaCl (2); Biotinylated Antibody(2)

Flash Tag RNA labeling

250 ng of total RNA was used as a starting material in the microRNA expression profiling experiment performed on the GeneChip miRNA 2.0 arrays (Affymetrix). Reactions were performed with the FlashTag Biotin HSR RNA Labeling Kit (Genisphere) according to the manufacturer's manual. At first 2µl RNA Spike Control Oligos were added to the total RNA. Next 1.5µl 10X Reaction Buffer, 1.5µl of 25mM MnCl2, 1.0µl of diluted ATP Mix, and

1.0µl PAP Enzyme were added to each RNA/Spike Control Oligos mix. During the 15 min. incubation at 37°C polyA - tails were added at the 3' end of RNA by the Poly(A)-Polymerase (PAP). In the next step the signal molecule biotin was ligated to the target miRNA sample. Four µl of 5X FlashTag Biotin HSR Ligation Mix and 2µl of T4 DNA Ligase were added to 15µl of tailed RNA and incubated for 30 min. at RT. The reaction was stopped by adding 2.5µl of HSR Stop Solution.

Hybridization

The following components: BSA, herring sperm DNA solution, biotinylated anti-streptavidin, Control B2- oligonucleotides, DMSO, formamide and the hybridization buffer were added to the biotin-labeled samples. The hybridization cocktail was incubated in 99°C for 5 minutes, and then in 45°C for 5 minutes. After injection onto the Affymetrix GeneChip, arrays were incubated for 16 h in 48°C with rotation at 60 rpm.

Washing, staining and detection

Microarrays were washed and stained in the Affymetrix GeneChip Fluidics Station 450. The FS450_0003 protocol was applied and wash buffer A, wash buffer B, array holding buffer, staining cocktails 1 and 2 were used. During this process streptavidin-phycoerythrin complexes bind to the biotin-labeled miRNAs. Next, the complexes are detected during scanning performed with Affymetrix GeneChip Scanner 3000, according to the manufacturer's protocol. As control served commercial human brain reference RNA (Ambion).

Quality control and normalization

A quality control was conducted with miRNA QC tool (Affymetrix). It was used for data summarization, normalization and probeset detection (greater than 2000). Quality control was performed using Spearman correlation of expression values between samples (higher than 0.5). The procedure of signature discovery was similar to the one used for the analysis of the Affymetrix U133 Plus 2.0 arrays.

2.7 STATISTICAL ANALYSES

Data handling and statistical analysis were carried out by GraphPad Prism 5 software for Windows (GraphPad Software Inc, San Diego, CA) using Chi-square test. A difference was considered statistically significant at p value < 0.05.

Chapter 3

Results

3.1 SELECTION AND ESTABLISHMENT OF THE MUTATION ANALYSIS METHOD

In order to determine mutation status of the genes most frequently mutated in colorectal cancer, a custom allele-specific PCR (Applied Biosystems) was conducted. The method was selected after a critical review of mutation detection technologies available at this time with respect to its sensitivity. Other advantages of this real-time PCR-based method were its easy handling, short time of analysis and relatively low cost. The allele-specific assays are, however, not suited for detecting *de novo* mutations. The target mutations need to be defined before the analysis. Moreover the method requires analysis of each selected nucleotide exchange in a separate reaction. In the population study, mutation analysis was therefore restricted to the KRAS, BRAF, and PIK3CA hotspots which were known to be commonly mutated in colorectal cancer patients. Allele-specific assays were designed for: 8 substitutions in the KRAS gene (34G>A/G12S, 34G>T/G12C, 34G>C/G12R, 35G>A/G12D, 35G>T/G12V, 35G>C/G12A, 38 G>A/G13D, and 436 G>A/A146T), the most frequent mutation in the BRAF gene (1799 T>A/V600E), and 3 hotspots in the PIK3CA gene (1624G>A/E542K, 1633G>A/E545K, 3140A>G/H1047R). Mutation status of additional hotspots in the APC gene (4348 C>T/R1450*), CTNNβ1 gene (121 A>G/T41A, 134 C>T/S45F) and in the TP53 gene (524 G>A/R175H, 742 C>T/R248W, 743 G>A/R248Q, 818G>A/R273H, 844 C>T/ R282W) was also determined in the panel of 67 samples used in the treatment experiment in addition to the KRAS, BRAF and PIK3CA analyses.

3.1.1 Titration of the Mutated Cell Line DNA in the Wild-type Genomic DNA

Serial dilutions of mutant cell lines with the wild-type genomic DNA were performed to estimate sensitivity of the allele-specific real-time PCR assays. DNA was extracted from cell lines, which harbour mutations of interest (Tab.3.1), and then mixed with the genomic DNA (gDNA), isolated from lymphocytes of peripheral blood system from healthy controls. Mutated cell line DNA was serially diluted with the wild-type gDNA in the following proportions: 1:1 (100% mutated cell line), 1:2 (50% mutated cell line), 1:5 (20% mutated cell line), 1:10 (10% mutated cell line), 1:20 (5% mutated cell line), 1:50 (2% mutated cell line), and 1:100 (1% mutated cell line) (an example of the titration result is shown in Fig.3.2) and analyzed by allele-specific real-time PCR. Sensitivity of the assays was evaluated for all the probes used in mutation analysis of KRAS, BRAF and PIK3CA and for the majority of the probes used in the analysis of additional genes (APC, CTNNB1, and TP53).

Tab.3.1. *Cell lines that served as positive in mutation analysis and the mutations that they harbour. Sensitivity (S+) - content of the mutant alleles in the sample required for reliable assessment* of *the mutation status*

Target gene	Target Mutation nt	Cell line	Cell line zygosity	S+ mutant alleles
KRAS	34 G>A	A549	Homozygous	10%
KRAS	34 G>T	NCI-H385	Heterozygous	1%
KRAS	34 G>C	HuP-T3	Heterozygous	5%
KRAS	35 G>A	A427	Heterozygous	2.5%
KRAS	35 G>T	SW620	Homozygous	2%
KRAS	35 G>C	RPMI-8226	Heterozygous	2.5%
KRAS	37 G>T	TOV 21	Heterozygous	2.5%
KRAS	38 G>A	LoVo	Heterozygous	5%
BRAF	1799 T>A	HT-29	Heterozygous	2.5%
KRAS	436 G>A	ML-2	Heterozygous	2.5%
PIK3CA	1624 G>A	T84	Heterozygous	1%
PIK3CA	1633 G>A	HCT-15	Heterozygous	5%
PIK3CA	3140 A>G	SKOV-3	Heterozygous	5%
CTNNB1	134C>T	-	-	-
CTNNB1	121A>G	A427	Homozygous	10%
TP53	524 G>A	KLE	Homozygous	10%
TP53	742 C>T	-	-	-
TP53	743 G>A	OVCAR-3	Homozygous	10%
TP53	818 G>A	SW620	Homozygous	5%
TP53	844 C>T	HuP-T3	Homozygous	5%
APC	4348 C>T	-	-	-

Results revealed a variety in the sensitivity of different assays from 1% mutant alleles to 10% mutant alleles required for reliable assessment of the mutation status. The cut-off for each of the assays is displayed in Tab.3.1. Such differences in the sensitivity result from the affinity of the probe to hybridize, which depends on the local sequence context. A general cut-off was set at 10% according to the assays with the worst performance limiting the sensitivity of the method.

3.1.1.2. Addition of the non-labeled oligonucleotides to avoid cross-reactivity of the imperfectly matched probes

Three cell lines: A427, SW620, and RPMI-8226 that carry different mutations in the KRAS codon 12 (nucleotide position 35 G>A, 35 G>T, and 35 G>C respectively) were analyzed using allele-specific real-time PCR (Fig.3.1). All three cell lines were analyzed with the same assay detecting mutation 35 G>A. This assay contains two probes. The probe that detects the mutation 35 G>A is labeled with FAM and another probe, which detects the wild-type is labeled with VIC. DNA of each cell line was serially diluted with the wild-type genomic DNA resulting in the 90%, 50%, and 10% tumour DNA vs. wild-type DNA. If the assays were specific, FAM signal would be expected only in the A427 cell line titration. Unfortunately, FAM signal was observed in all three cell line titrations (Tab.3.2). In the dilution containing 90% of cell line alleles, the FAM fluorescence signal observed during the analysis of the unspecific cell lines constituted app. 40% and 75% of the specific signal for the SW620 and RPMI-8226 respectively. The unspecific signal resulting from the analysis of further dilutions of the RPMI-8226 with the wild-type DNA was even higher than the signal detected in the corresponding dilutions of the specific cell line. The unspecific signal was a result of the cross-reactivity of the imperfectly matched probes (Fig. 3.1 A). To silence this unspecific signal, unlabeled oligonucleotides competing with the unmatched probes were added to the reactions. First the oligonucleotide blocking 35 G>T and G>C were tested in separate reactions (Fig.3.1 B and C). Various oligonucleotide concentrations and lengths were tested in order to avoid the unspecific signal, but at the same time not to decrease the specific signal from the matched probe (data not shown). Finally an equimolar mixture of both blocking oligonucleotides was added to the reaction (Fig.3.1 D). The non-labeled oligonucleotides prevented the generation of unspecific fluorescence signals. Mean absolute fluorescent values of FAM and VIC for the three cell lines are displayed in Tab.3.2.

Possible sequence variations in the KRAS nt position 35: AACTTGTGGTAGTTGGAGCTG **[G/A/T/C]** TGGCGTAGGCAAGAGTGCCTT

Probe detecting wild-type sequence: TTGGAGCTG**G**TGGCGTA

Cell line A427 (35 G>A) sequence: TGTGGTAGTTGGAGCTG**A**TGGCGTAGGCAAGA

Probe detecting 35 G>A: TTGGAGCTG**A**TGGCGTA

Cell line SW620 (35 G>T) sequence: TGTGGTAGTTGGAGCTG**T**TGGCGTAGGCAAGA

Oligo blocking 35 G>T: TAGTTGGAGCT**T**GTGGCGTAG

Cell line RPMI-8226 (35 G>C) sequence: TGTGGTAGTTGGAGCTG**C**TGGCGTAGGCAAGA

Oligo blocking 35 G>C: TAGTTGGAGCT**C**GTGGCGTAG

Tab.3.2. *Mean absolute fluorescent values of FAM (mutant - label) and VIC (wild-type - label) in the experiment testing an assay, which detects KRAS mutation 35 G>A in three different cell lines: A427 (specific; mutant 35 G>A); RPMI-8226 (unspecific: mutant 35 G>C); SW620 (unspecific: mutant 35 G>T). Three different dilutions of the cell line with the wild-type DNA were tested: 90%, 50% and 10% under four different conditions: A) Standard - no oligo added to the reaction; B) Oligo blocking 35 G>T added to the reaction C) Oligo blocking 35 G>C added to the reaction D) Mix of the two oligo added to the reaction. Ratio - of the FAM fluorescence signal after addition of the blocking oligonucleotides in comparison to the FAM fluorescence signal of without blockers*

Cell line		**A. No oligont.**		**B. + 35 G>T blocker**			**C. + 35 G>C blocker**			**D. + 2 blockers mix**		
%	**Mutation**	**FAM**	**VIC**	**FAM**	**VIC**	**Ratio (FAM)**	**FAM**	**VIC**	**Ratio (FAM)**	**FAM**	**VIC**	**Ratio (FAM)**
A427 10%	35 G>A	0,63	2,32	0,56	2,15	89%	0,51	2,18	81%	0,57	2,15	90%
A427 50%	35 G>A	1,75	2,00	1,82	1,89	104%	1,87	1,85	107%	2,02	1,80	116%
A427 90%	35 G>A	3,13	1,65	3,17	1,56	101%	3,01	1,53	96%	3,16	1,59	101%
RPMI-8226 10%	35 G>C	0,74	2,18	0,38	2,01	51%	0,16	1,88	21%	0,21	1,80	29%
RPMI-8226 50%	35 G>C	1,85	1,87	1,00	1,77	54%	0,34	1,60	19%	0,36	1,64	19%
RPMI-8226 90%	35 G>C	2,32	1,84	1,27	1,62	55%	0,40	1,47	17%	0,49	1,57	21%
SW620 10%	35 G>T	0,60	2,23	0,16	1,90	26%	0,36	1,87	59%	0,27	1,99	44%
SW620 50%	35 G>T	1,07	2,02	0,41	1,53	38%	0,70	1,58	66%	0,44	1,43	42%
SW620 90%	35 G>T	1,21	2,01	0,54	1,26	45%	0,73	1,50	61%	0,56	1,25	46%
Neg. control	-	0,18	2,26	0,18	2,26	-	0,15	2,19	-	0,17	2,24	-
NTC	-	0,01	0,16	0,01	0,26	-	0,01	0,17	-	0,02	0,06	-

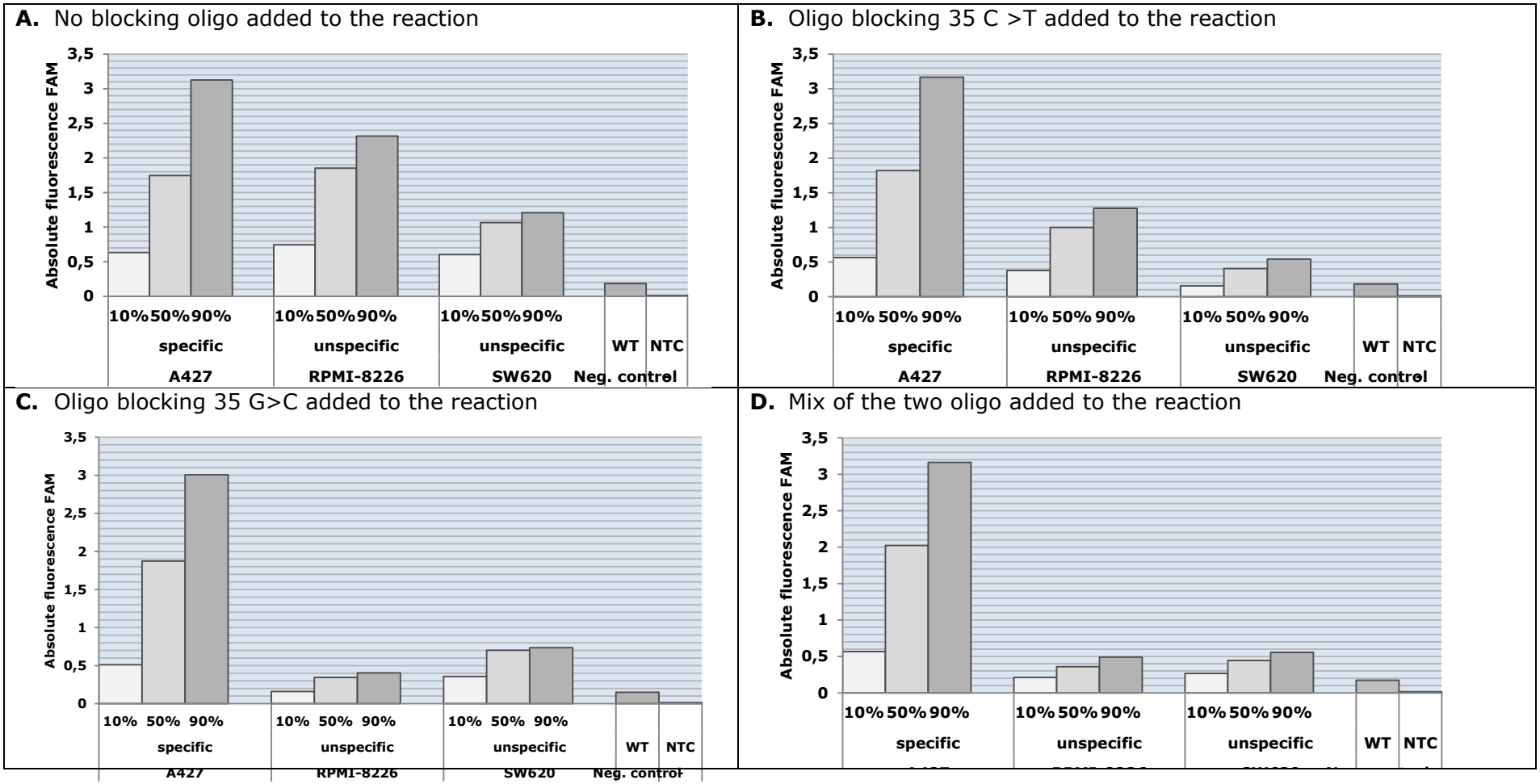


Fig.3.1. *Silencing the unspecific signal by blocking oligonucleotides. Mean absolute fluorescent values of FAM (mutant - label) in the experiment testing an assay, which detects KRAS mutation 35 G>A in three different cell lines: A427 (specific); RPMI-8226 (mutant 35 G>C); SW620 (mutant 35 G>T). Three different dilutions of the cell line in the wild-type DNA were tested: 90%, 50% and 10% under four different conditions:*

A) Standard - no oligont added to the reaction; B) Oligont blocking 35 G>T added to the reaction C) Oligo blocking 35 G>C added to the reaction D) Mix of the two oligont added to the reaction

3.1.2. Comparison of the Sensitivities of the Allele-specific Real-time PCR Assays with Dideoxy Sequencing

The homozygous SW620 cell line, which harbours G12V KRAS mutation, was mixed with the wild-type gDNA to obtain 50%, 20%, 10% and 5% tumour DNA content. Mutation status of these samples (titration) was analyzed by allele-specific real-time PCR assays and conventional sequencing (Fig.3.2). Allele-specific PCR allowed detection of 2% mutant alleles for this specific KRAS assay, while dideoxy chain terminator sequencing yielded a sensitivity of 10 - 20%.

3.1.3 Comparison of Matched FFPE and Snap-frozen Tissues

Two types of differently preserved tumour tissue specimens were used in the mutation study - FFPE and snap-frozen tissues. In order to estimate the variance of the assay performance for both types of material, a pilot experiment was conducted in 50 match pairs of snap-frozen and FFPE derived DNA samples. KRAS mutation status in codon 12 was analyzed using two different assays detecting mutations G12D (sensitivity 5%) and G12V (sensitivity 2%). 10 mutations (seven G12D and three G12V mutations) were detected in both snap-frozen and FFPE derived DNA samples from the 50 matched-pairs. These results suggest that both -FFPE blocks and fresh frozen tissue specimens can be used with allele-specific real-time PCR. Therefore, in most of the analyses, I proceeded with FFPE-DNA as FFPE remains the standard tissue in routine clinical practice.

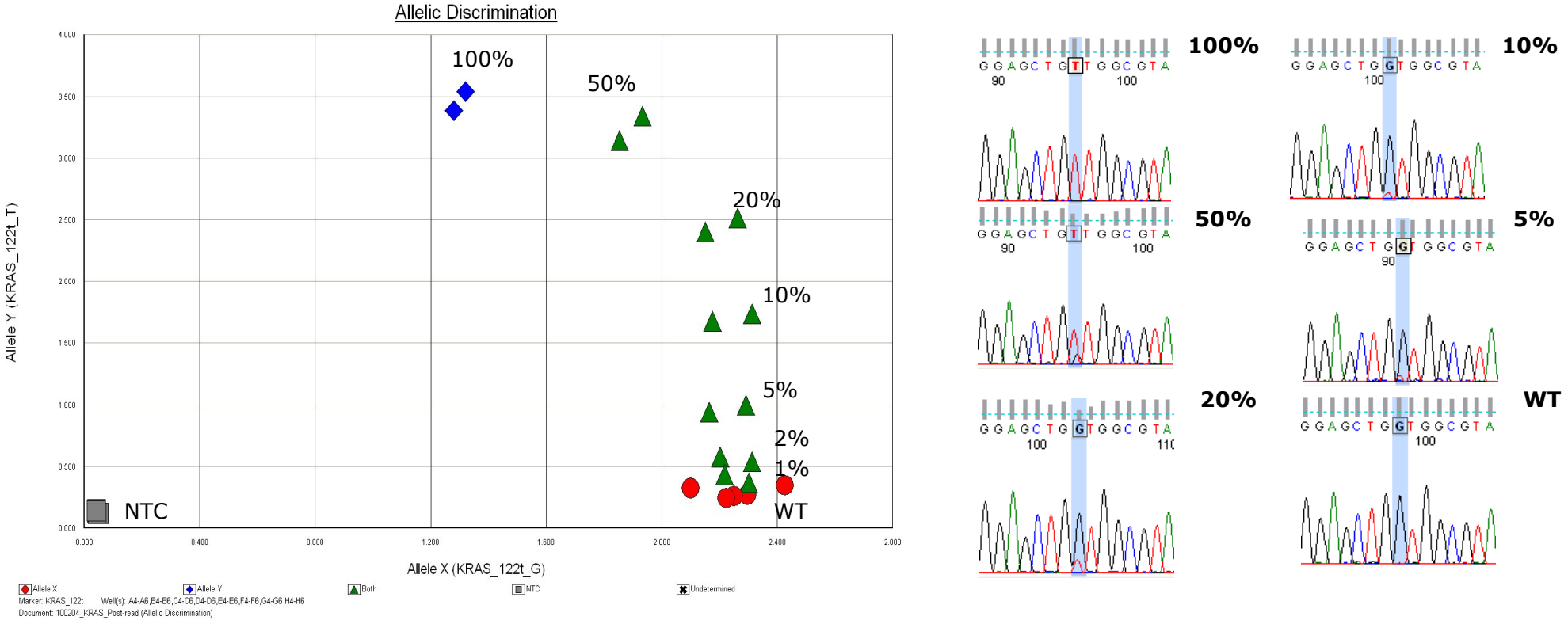


Fig.3.2. *Comparison of the results obtained by mutation analysis performed by allele-specific real-time PCR and direct sequencing; titration was performed by serial dilutions of the homozygous mutant cell line SW620 (KRAS 35 G>T) with the KRAS wild-type genomic DNA and mimics different tumour cell content in the sample. On the left: allele-specific real-time PCR X-Y bivariate plot of fluorescence of VIC (G allele, wild-type) and FAM (T allele, mutant). Each colored symbol in each panel represents a single sample; every sample was analyzed in duplicate. Red dot: homozygous wild-type; blue diamond: homozygous mutant; green triangle: mixture of mutant cell line SW620 with KRAS wild-type genomic DNA; grey squares: non-template controls (NTC). On the right: chromatograms obtained by sequencing of particular dilutions*

3.2 THE COHORT OF 864 CRC PATIENTS AS A REPRESENTATION OF THE CRC POPULATION IN GERMANY

The chi-square test was used to assess the distribution of categoric variables within the analyzed population of the patients. The results were compared with the statistical data from the Tumour Register of the Land Brandenburg (http://www.tumorzentrum-brandenburg.de), which includes all CRC cases registered between 2000 and 2009 in state Brandenburg. Statistical analysis revealed similar distribution of gender (p=0.2117; OR: 1.093; 95% CT: 0.9507 to 1.256) and age (p=0.5009; OR: 1.076; 95% CT: 0.8697 to 1.331). There was a prevalence of colon samples in both populations, stronger in the MSKK/RVS study patients (60% vs. 66%; p=0.0011). The 864 samples had the following clinical stage distribution: UICC stage I - 18%, stage II - 33%, stage III - 41% and stage IV - 8%. The stage distribution in the Tumour Register of the Land Brandenburg is: UICC stage I - 22%, stage II - 27%, stage III - 27% and - 18% in stage IV. The difference in the distribution of UICC stages is statistically significant (p<0.0001; Chi-square df: 119.6, 3) and is a result of different classification criteria. In the MSKK as well as in the RVS study, only patients with histologically confirmed metastases are registered as stage IV, which is not the case in public statistics. Therefore a smaller fraction of stage IV in our studies (8% vs. 18%) is compensated by higher frequency of patients registered with stage III CRC (41% vs. 27%).

3.2.1 Mutation Distribution in KRAS, BRAF and PIK3CA in 864 Patients

Mutation status of three selected oncogenes KRAS, BRAF, and PIK3CA was assessed in this subpopulation of 864 patients. The most frequently mutated hotspots that are known to contribute to tumourigenesis and resistance to targeted therapies in colorectal cancer were selected for the analysis. According to the COSMIC database 9 selected hotspots in KRAS gene cover 99% of all mutations found in KRAS in CRC patients, the V600E mutation covers over 98% of all substitutions in the BRAF, and 3 hotspots analyzed in PIK3CA account for 77% of mutations in this gene (Tab.3.3.). If no mutation was found in analyzed hotspots, the sample was considered wild-type for the analyzed gene.

Six hundred and nine (70%) samples were extracted from FFPE tissues and 255 (30%) samples from snap-frozen material. In FFPE samples mutations were detected in 38% (KRAS), 10% (BRAF), and 15% (PIK3CA) of the analyzed samples while in snap-frozen tissue KRAS mutations were found in 45%, BRAF in 8% and PIK3CA in 15% of the samples (Tab.3.4). There was no significant difference in the frequency of mutations between the two tissue types (p=0.1731, OR: 1.241; 95% CI: 0.9093 -1.694).

Tab.3.3. *Comparison of mutation frequencies between the Catalogue of Somatic Mutations in Cancer (COSMIC) and MSKK/RVS study*

Gene:	Mutations positions:		COSMIC database		Fraction of all mut.	MSKK/RVS study	
	nt	aa	Frequency	Fraction		Frequency	Fraction
KRAS	34 G>A	G12S	2.2%	6.1%	99%	2.8%	6.9%
	34 G>T	G12C	3.0%	8.4%		2.3%	5.7%
	34 G>C	G12R	0.5%	1.3%		0.2%	0.6%
	35 G>A	G12D	12.5%	34.5%		12.6%	31.2%
	35 G>T	G12V	8.0%	22.1%		9.8%	24.4%
	35 G>C	G12A	2.3%	6.4%		2.4%	6.0%
	37 G>T	G13C	0.2%	0.5%		0.1%	0.3%
	38 G>A	G13D	7.1%	19.6%		8.0%	19.8%
	436 G>A	A146T	0.1%	0.3%		2.2%	5.4%
BRAF	1799 T>A	V600E	10.1%	98%	98%	9.5%	100%
PIK3CA	1624 G>A	E542K	2.2%	18%	77%	3.1%	20.9%
	1633 G>A	E545K	4.1%	33%		7.9%	52.7%
	3140 A>G	H1047R	3.2%	26%		4.3%	28.7%

Using the allele-specific assays described in chapter 2, altogether at least one mutation was detected in 462 out of 864 patients (53%). Most of the mutations were G <-> A transitions (63%), while different transversions account for remaining 37% of the mutations. The majority of mutations were observed in the KRAS gene and account for more than 60% of all mutations that were found. There were 348 patients (40%) who carried mutations in this gene, in two cases double KRAS mutations were detected in the same patient (G12C + G12D/ G12S + A146T). Seventy-five percent of all KRAS mutations were found in codon 12, 20% in codon 13, and 5% in codon 146. The most common mutations was G to A substitution in nucleotide position 35, which affects codon 12 and results in amino acid change of glycine to aspartic acid (over 30% of all KRAS mutations). The second most frequent mutation was G to T exchange in the same position, which results in amino acid substitution of glycine to valine and accounts for 24% of KRAS mutations. Another common KRAS mutation was a substitution 38 G>A, which leads to amino acid change of glycine to aspartic acid in codon 13 (20% of all KRAS mutations). The distribution of different KRAS mutations in the analyzed hotspots is shown in Fig.3.3.

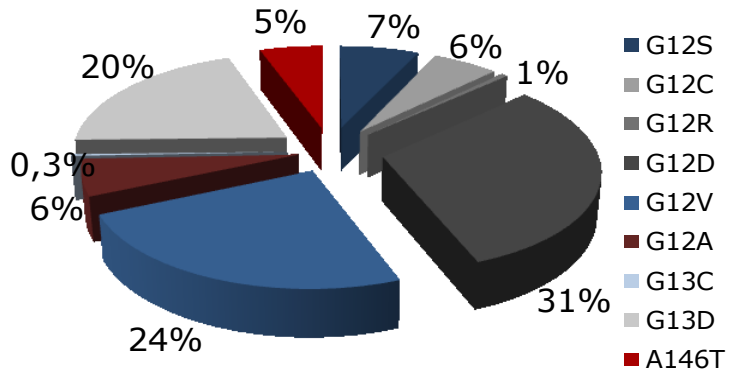


Fig.3.3. *Distribution of different substitutions among all KRAS mutations detected in the group of 864 CRC patients. The majority of mutations were detected in codon 12 (75%), 20% of mutations were found in codon 13 and remaining 5% in codon 146 of the KRAS gene*

The frequency in the majority of identified mutations detected among the 864 CRC patients is in agreement with the data reported in COSMIC database (Tab.3.3). Significant difference was found only in codon 146 ($p<0.0001$). Our results indicate that KRAS A146T substitution occurs with a frequency of over 2% among CRC patients, while the frequency reported in COSMIC is 0.1%.

BRAF mutations were analyzed in one hotspot (V600E) and were found in 82 patients (9.5%). This finding stays in concordance with the frequency reported in COSMIC database (10%). BRAF mutations were mutually exclusive with KRAS mutations ($p\leq 0.0001$) in 80 cases. Two patients showed mutations in both BRAF and KRAS genes. Both KRAS mutations coexisting with mutations in BRAF were substitutions in codon 13 (G13D). One of the two patients had additionally a mutation in the PIK3CA gene.

Mutations in the PIK3CA gene were assessed in two hotspots in exome 9 (E542K; E545K) and one hotspot in exon 20 (H1047R). Altogether alterations in at least one of the PIK3CA hotspots were detected in 129 (15%) of the patients. Mutations in exon 9 were found in 94 (11%) and in exon 20 in 37 (4%) of analyzed patients. The most common mutation was a substitution in nucleotide position 1633 that affects amino acid codon 545 and leads to a change of glutamic acid to lysine. It accounts for more than 50% of all mutations detected in PIK3CA in our setting. The frequency of this mutation is substantially higher than reported in COSMIC (Tab.3.3). In three cases two different PIK3CA mutations were found in the same tumour (E545K + E542K (P573) / H1047R (P172; P267)).

Tab.3.4. *Comparison of mutational distribution between the samples isolated from the FFPE and from the snap-frozen material in the framework of the MSKK and RVS studies*

Gene:	Mutations positions:		FFPE		Snap-frozen	
	nt	aa	Mutation No	%	Mutation No	%
KRAS	34 G>A	G12S	15	2,5%	9	3,5%
	34 G>T	G12C	14	2,3%	6	2,4%
	34 G>C	G12R	1	0,2%	1	0,4%
	35 G>A	G12D	75	12,3%	34	13,3%
	35 G>T	G12V	54	8,9%	31	12,2%
	35 G>C	G12A	15	2,5%	6	2,4%
	37 G>T	G13C	1	0,2%	0	0,0%
	38 G>A	G13D	48	7,9%	21	8,2%
	436 G>A	A146T	12	2,0%	7	2,7%
BRAF	1799 T>A	V600E	62	10,2%	20	7,8%
PIK3CA	1624 G>A	E542K	22	3,6%	5	2,0%
	1633 G>A	E545K	45	7,4%	23	9,0%
	3140 A>G	H1047R	25	4,1%	12	4,7%

There were 96 cases, which had more than one mutation in the analyzed hotspots of the three genes: KRAS, BRAF and PIK3CA. In 81 cases mutations in the KRAS gene occur concomitantly with mutations in PIK3CA. BRAF and PIK3CA mutations were detected in same tumour tissue in only 13 cases. In two cases mutations in BRAF and KRAS were found to coexist in the same tumour sample (P224; P286). One of these patients had mutations in all three analyzed genes (P286). Detailed information about the mutation status of KRAS, BRAF and PIK3CA in individual patients is displayed in Tab.S5.

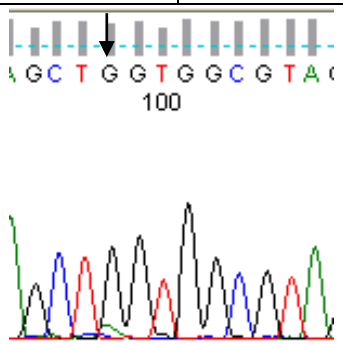
3.2.2 Sanger Dideoxy Sequencing and Real-time PCR in Matched 304 Samples

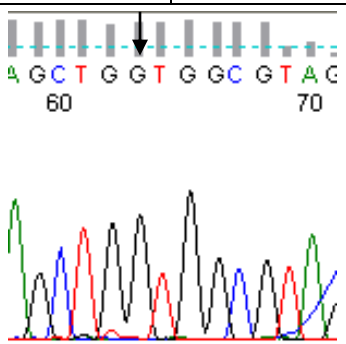
A subset of 304 samples was analyzed for KRAS mutation by both allele-specific real-time PCR and Sanger dideoxy-sequencing. The PCR sequencing primers spanned exon 2 of the KRAS gene allowing detection of mutations in codon 12 and 13. Sequencing results of 21 samples were of poor quality. Nineteen of these samples were extracted from the FFPE tissue and two from the snap-frozen material. Six of the low quality samples were excluded due to the unincorporated dye terminators that formed a dye-blob and hampered the interpretation, while the rest of the exclusions resulted from a high background noise in the chromatograms of the analyzed samples. Analysis of the remaining 283 samples showed five (2%) discrepancies between the two methods (P9; P60; P226; P381; P461). Five mutations were found by real-time PCR, but either there was no signal from the mutated allele on the sequencing chromatogram or the signal was so weak that a prospective interpretation was impossible. Altogether dideoxy sequencing failed in 26 cases (21 cases of poor quality and in 5 cases the mutations was not found). In 112 samples identical mutations were found by real-time PCR as well as by direct

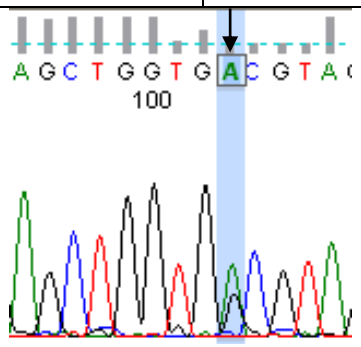
sequencing. An example of the comparison of the dideoxy-sequencing versus allele-specific real-time PCR performance in mutation analysis is shown in Tab.3.5. All the 117 chromatograms showing mutations or discrepancies between the two methods are displayed in the supplementary table S6. The remaining 166 samples were wild-type in KRAS codon 12 and 13 as shown by allele specific real-time PCR and by dideoxy sequencing. Ten examples of the wild-type chromatograms are also shown in Tab.S6.

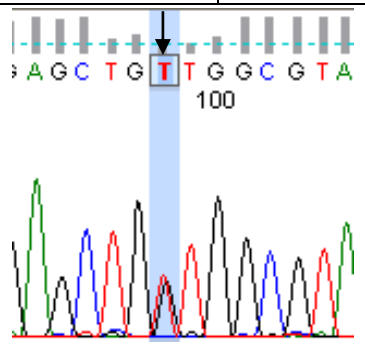
Tab.3.5. *Example of the mutation analysis performed using allele-specific real-time PCR assays and the dideoxy-sequencing. The FAM (mutant probe)/VIC (wild-type probe) delta Rn ratios indicate the magnitude of the signal generated in the PCR reactions. The Rn values are displayed for the analyzed samples in the first row. Below the Rn values of the controls are presented: negative (wild-type DNA); positive (mutant: 0.5%, 1%, 2%, 5%, 10%, 20%, 50% and 100%). As positive controls served cell lines that harbour investigated mutations: A549- 34 G>A (homozygous); A427- 35 G>A (heterozygous); LoVo - 38 G>A (heterozygous); SW620 - 35 G>T (homozygous) that were serially diluted with the wild-type DNA. Red font underlines the sensitivity threshold for each used cell line. Below the table, corresponding chromatograms are shown for the analyzed samples. In sample A and B mutation found by real-time PCR (FAM/VIC ratio >cut-off) could not be detected by sequencing. In samples C and D the mutations could be found by both methods*

A.		B.		C.		D.	
Sample	FAM/VIC	Sample	FAM/VIC	Sample	FAM/VIC	Sample	FAM/VIC
P60	0,85	P381	0,87	P93	2,57	P833	2,24
WT	0,21	WT	0,10	WT	0,21	WT	0,11
A549_0.5%	0,29	A427_0.5%	0,09	LoVo_0.5%	0,20	SW620_0.5%	0,17
A549_1%	0,29	A427_1%	0,09	LoVo_1%	0,23	SW620_1%	0,19
A549_2%	0,35	A427_2%	0,15	LoVo_2%	0,26	SW620_2%	0,23
A549_5%	0,41	A427_5%	0,18	LoVo_5%	0,37	SW620_5%	0,49
A549_10%	0,44	A427_10%	0,32	LoVo_10%	0,46	SW620_10%	0,77
A549_20%	0,53	A427_20%	0,49	LoVo_20%	0,98	SW620_20%	1,12
A549_50%	0,87	A427_50%	1,00	LoVo_50%	1,84	SW620_50%	2,03
A549_100%	16,36	A427_100%	2,49	LoVo_100%	3,72	SW620_100%	5,45









3.2.3 Mutation Status vs. Clinicopathological Characteristics of the Patients

Next, the mutation status was analyzed in relation to different clinical and pathological characteristics such as gender and age of the patient, localization, morphology and stage of the tumour, or number of the lymph nodes in which regional metastases were found (Tab.3.6). The mutation status was not analyzed in the context of the outcome of the patients as a 5-year follow-up was not yet available. Chi-square test was applied to verify the relation of the various clinical and histological characteristics and the mutation status of KRAS, BRAF and PIK3CA. KRAS mutations showed no relation to gender, age, tumour location, UICC-stage, lymph node invasion or morphology of the tumour. In contrast, BRAF-mutations were significantly associated with female gender ($p<0.0001$; OR: 0.2673; 95% CI: 0.1626 - 0.4396) and tumour location in the colon ($p<0.0001$; OR: 6.087; 95% CI: 2.766 - 13.40). In addition, there was a slight but statistically not significant association between BRAF mutations and older age (> 60yrs) ($p= 0.0632$; OR: 0.5281; 95% CI: 0.2665 - 1.046), and number of positive lymph nodes ($p=0.0668$; Chi square df: 7.164, 3). Patients, who carried PIK3CA mutations were more likely to have colon cancer than rectal cancer ($p=0.0569$; OR: 1,502; 95% CI: 0.9861 - 2.287) although this association did not reach statistical significance.

Tab.3.6. *Clinical and pathological features of 864 patients with respect to the mutation status analyzed in KRAS, BRAF and PIK3CA gene*

Characteristic	Total	%	Any Mut	%	BRAF Mut	%	KRAS Mut	%	PIK3CA Mut	%	> 1 Mut	%
Total n pts	864	100%	462	53%	82	9%	348	40%	129	15%	96	11%
Gender												
Male	493	57%	239	48%	24	5%	196	40%	71	14%	52	11%
Female	361	42%	217	60%	58	16%	147	41%	57	16%	44	12%
X	10	1%	6	-	0	-	5	-	1	-	0	-
Age												
≤60 yrs old	172	20%	79	46%	10	6%	61	35%	22	13%	14	8%
>60 yrs old	688	80%	381	55%	72	10%	286	42%	106	15%	82	12%
X	4	0%	2	-	0	-	1	-	1	-	0	-
Localization												
Colon	569	66%	323	57%	74	13%	228	40%	94	17%	72	13%

Rectum	292	34%	136	47%	7	2%	119	41%	34	12%	24	8%
Characteristic	Total	%	Any Mut	%	BRAF Mut	%	KRAS Mut	%	PIK3CA Mut	%	> 1 Mut	%
X	3	0%	3	-	1	-	1	-	1	-	0	-
UICC Stage												
I	154	18%	79	51%	14	9%	58	38%	26	17%	19	12%
II	283	33%	137	48%	20	7%	103	36%	46	16%	32	11%
III	353	41%	206	58%	41	12%	157	44%	44	12%	35	10%
IV	69	8%	37	54%	7	10%	28	41%	11	16%	9	13%
X	5	1%	3	-	0	-	2	-	2	-	1	-
No Lymph nodes pos.												
0	439	51%	217	49%	34	8%	162	37%	73	17%	52	12%
1-3	203	23%	116	57%	19	9%	90	44%	31	15%	23	11%
4-6	97	11%	54	56%	11	11%	41	42%	12	12%	10	10%
7 or more	106	12%	65	61%	17	16%	47	44%	10	9%	9	8%
X	19	2%	10	-	1	-	8	-	3	-	2	-
Morphology												
Adenocarcinoma	623	72%	308	49%	41	7%	245	39%	81	13%	58	9%
Mucinous carcinoma	70	8%	55	79%	15	21%	37	53%	18	26%	15	21%
Mixed diff.with mucin	44	5%	39	89%	9	20%	27	61%	11	25%	8	18%
Rest after neoadj.th.	25	3%	14	56%	0	0%	12	48%	4	16%	2	8%
Synchronous CRC	42	5%	13	31%	3	7%	9	21%	4	10%	3	7%
Tumour in adenoma	19	2%	12	63%	4	21%	8	42%	6	32%	6	32%
Other diff.	30	3%	15	50%	7	22%	7	28%	3	9%	2	7%
Unusual diff.	11	1%	6	55%	3	25%	3	25%	2	17%	2	18%

3.3 CHARACTERISATION OF THE PANEL OF 133 COLORECTAL CANCERS XENOGRAFT MODELS

3.3.1 Results of the Engraftment of 239 Patient-derived Tumour Specimens

A total of 239 CRC primary tumour specimens were transplanted subcutaneously into immunodeficient mice. Transplanted samples derived from the patients of all four UICC stages enrolled into the prospective multicenter MSKK study. Out of the 239 tumour specimens, 149 passagable xenograft models, deriving from 144 patients, were established, resulting in the engraftment rate of 62%. Altogether 90 samples failed to engraft. In 22 cases the reason for a failure was an inflammation or an abscess and in remaining 68 cases the reason was unknown. Histological analysis and quality control (QC) of the clinical data of the 149 models led to the exclusion of 16 xenografts. Some of the engrafted samples turned out to be adenomas or metastases originated from other primaries. Tumour specimens from the patients who received neoadjuvant treatment prior to surgery or who withdrew their informed consent account for remaining exclusions. Finally 133 high-quality xenograft models derived from primary tumours of 130 chemonaive patients remained after assessing the quality control.

3.3.2 Morphological Entity between Original Patient Tumours and Xenografts

Histological examination was performed to compare the properties of established xenografts with the matching primary tumours. For the majority of the samples a high morphological similarity between original patient carcinoma and the xenografts derived thereof was observed (Fig.3.4). Hundred twenty-two cases out of 133 were classified identically in respect to their histological type. In 11 cases differences were observed between the xenograft and matching primary patients' tissue. In six cases mucinous carcinoma was observed in xenografts and adenocarcinoma in primary tumours, while in three cases – in contrary – mucin reported in primary tumour, was no longer observed in the matching xenograft. In one case medullary differentiation was observed in the primary tumour, while after passaging mixed differentiation with mucin was reported. Finally, one adenoma of primary tumour differentiated into squamous cell carcinoma (data not shown).

Sixty seven models were passaged in order to perform drug testing. Each tumour tissue was further passaged onto twenty animals out of which five served as controls and were examined by the pathologist after the treatment experiment. Differences in the histological classification between the first set of passages and further passaged models used in the treatment experiment were observed in 8 models (data not shown). In one case adenocarcinoma differentiated into both adenocarcinoma and mixed differentiation

with mucin, in another case - mixed differentiation with mucin grew further as adenocarcinoma and undifferentiated tumour. In six cases mucinous tumours that were observed in early passages differentiated into adenocarcinoma. Four of these gained back the histological properties of primary tumours.

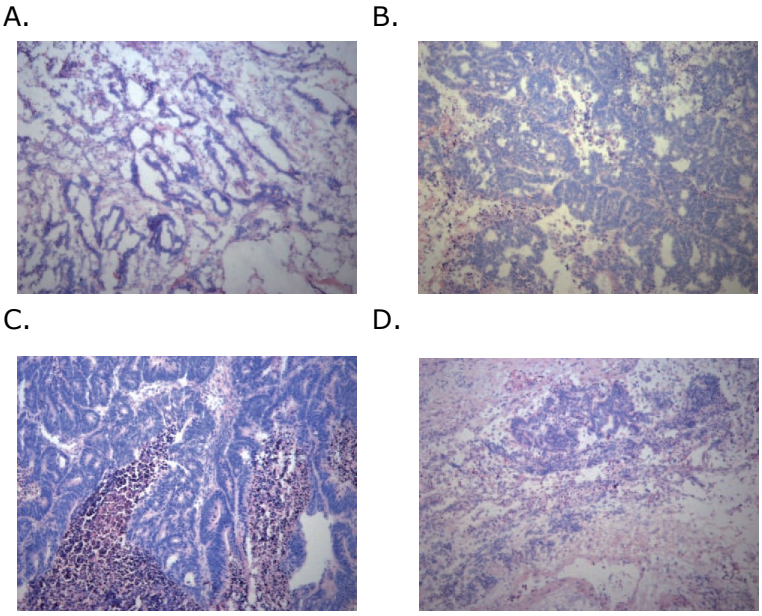


Fig.3.4. *Two examples of histopathological evaluation and comparison of patient tumour (on the left) and the xenograft derived thereof (on the right). Both xenografts show a denser atypical gland packing in comparison to the original adenocarcinoma. Xenograft B shows extensive necroses (photo: Dr. Irina Klaman)*

3.3.3 Gene Expression Profiling in Original Patient Tumour and Matched Xenograft

As a part of internal quality control of the xenografts entity between primary tumours and the xenografts was analyzed as well on the RNA expression level. Analysis was conducted using 254 samples, consisting of 127 pairs, each composed of an original sample of a primary colorectal tumour and its xenograft. Gene expression profiling revealed that primary tumours and their matched xenografts were more similar to each other (Spearman correlation of 0.80) than pairs of primary tumours (Spearman correlation of 0.66) or pairs of xenograft tissues (Spearman correlation of 0.69) (data not shown).

3.3.4 Mutations of KRAS, BRAF and PIK3CA in the Panel of 133 Models

In order to characterize the panel of 133 established xenograft models, mutation status was assessed in the most frequently mutated hotspots of KRAS, BRAF and PIK3CA. In three patients diagnosed with synchronous cancer two different tumour samples were engrafted. Mutation analysis did not reveal differences in mutation status in any of them, reducing the number of xenograft models for further statistical analysis to 130. At least one mutation was found in 69 (53%) of the analyzed models (Tab.S7). Forty four mutations (34%) were reported in codons 12, 13 or 146 of the KRAS gene. Seventeen models (13%) harboured BRAF substitution V600E. The same number of models was

found to carry PIK3CA mutations with the majority of them (14) found in exon 9. Only three models harboured PIK3CA alterations of exon 20 (2%). In 11 out of 17 cases that harboured PIK3CA substitutions, additional mutation was observed in KRAS (9 cases) or BRAF (2 cases). In one of the models mutation status of PIK3CA could not be assigned. KRAS and BRAF mutations were mutually exclusive. Mutation frequency in the MSKK/RVS study and in the xenografts was compared in order to evaluate how well the panel of 133 established models represents the population of patients. Altogether there was no difference in the overall mutation frequency in KRAS, BRAF, and PIK3CA (p=0.6804, OR: 0.9254; 95% CI: 0.6398 - 1.339) between the patients' population and the panel of 133 xenografts. A lower frequency of KRAS mutations of 34% observed in the xenograft models versus 40% in the MSKK/RVS study was compensated by BRAF mutations (13% - xenograft vs. 9% - MSKK/RVS) that occur only in KRAS wild-type patients. The differences in the mutation frequencies in KRAS and BRAF were, however, not statistically significant (KRAS: p=0.1171, OR: 1.365, 95% CI: 0.7329 - 1.082; BRAF: p=0.2030, OR: 0.670, 95% CI: 0.3988 - 1.218; PIK3CA: p=0.5778, OR: 1.167, 95%CI: 0.6776 - 2.008).

3.4 TREATMENT EXPERIMENT WITH CETUXIMAB, BEVACIZUMAB AND OXALIPLATIN IN THE 67 XENOGRAFT MODELS

3.4.1 Response toward Single-agents: Cetuximab, Bevacizumab and Oxaliplatin

A panel of 67 out of the 133 xenograft models was used to assess the antitumour efficacy of three anti-cancer compounds - cetuximab, bevacizumab and oxaliplatin. Each of 67 tumour models used in the treatment experiments was engrafted onto 20 mice - 5 were used for testing of each therapeutic agent, and 5 served as non-treated controls. Cetuximab, bevacizumab and oxaliplatin were applied as single-agents (detailed treatment scheme: Tab.3.7). Responders were defined by T/C ratio (volume of the treated tumour in relation to the non-treated control) of <20%. Non-responder (resistant) models were defined with T/C ratios greater than 20%.

Tab.3.7. *Treatment scheme and response rates of the 67 pharmacologically characterized xenograft models. Treatment groups consist of 15 and control group of 5 animals each.*

Drug	Treatment scheme	Responder T/C < 20%	
		Number	%
Oxaliplatin (Eloxatin; Sanofi-Aventis)	qd 1-5; 5 mg/kg/d, i.p.	4/67	6%
Cetuximab (Erbitux; Merck)	qd 7x2; 50 mg/kg/d, i.v.	18/67	27%
Bevacizumab (Avastin; Genentech Inc.)	qd 4; 5 mg/kg/d, i.p.	2/67	3%

Altogether 20 models (30%) responded to at least one of the three therapeutic agents. Eighteen of 67 models (27%) showed a strong response toward cetuximab monotherapy. In contrast, four models (6%) responded to oxaliplatin and only two models (3%) responded to bevacizumab. One of the models responded to all three drugs and in two models response toward two drugs - cetuximab and oxaliplatin was observed. Detailed information on the response to oxaliplatin, cetuximab, and bevacizumab is shown in Tab.3.10. A sufficient number of responders were observed only toward single-agent cetuximab. Most of the further analyses are, therefore, limited to results of the treatment experiment with cetuximab.

In six models very strong response to cetuximab resulted in almost complete regression of the tumour (T/C <5%), further six models responded with reduction in size to 5 - 10% of the non-treated control tumour volume, and remaining six responded with a T/C ratio of 10 - 20%. Among 49 cetuximab non-responders (73%) there were 8/49 models with T/C ratio between 20% - 35%, 12/49 with T/C ratio of 35% - 50% and 29/49 non-responders with T/C ratio greater than 50% (Tab.3.8).

Tab.3.8. *Distribution of T/C (treated to control) ratios under the treatment with cetuximab in the group of 67 xenograft models*

Symbol	T/C Ratio	Class	No of models	%
-	> 50	Non-responder	29	43%
+	35 - 50	Non-responder	12	18%
++	20 - 35	Non-responder	8	12%
+++	5 - 20	Responder	12	18%
++++	< 5	Responder	6	9%
Total			n = 67	100%

Out of 18 events of response to cetuximab 9 occur in the xenograft models originating from male patients (n=31) and 9 from female patients (n=36). Among the models derived from the patients of 60 years old or younger, there were four responders (4/15), while remaining 14 responders represented patients of more than 60 years old (14/52). 46 out of 67 models were derived from colon cancer and 21 from rectal cancer patients. Rectal tumours were more likely to respond toward cetuximab than colon tumours (38% vs. 22%). There were 2/8 cetuximab responders of stage I (25%), 8/22 of stage II (36%), 4/28 stage III (14%), and 4/9 stage IV (44%; see Tab.3.9).

3.4.2 Mutation Distribution in the 67 Xenograft Models

Altogether at least one KRAS, BRAF or PIK3CA mutation was observed in 41 models (61%) out of this subset of 67 xenografts (Tab.3.9.). There were 35 single mutations observed: 27 in KRAS, 7 in BRAF and 1 in PIK3CA. In 6 cases (9%) mutations in two genes occur concomitantly in KRAS and PIK3CA. Majority of the KRAS mutations (23) were observed in codon 12 (70%), 6 mutations were found in codon 13 (18%), and 2 mutations in each - codon 61 (6%), and codon 146 (6%). Five mutations were detected in exon 9 and two in exon 20 of PIK3CA (Fig.3.5).

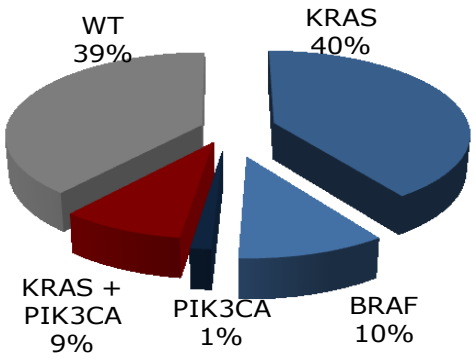


Fig.3.5. *Distribution of mutations detected in the hotspots of the KRAS, BRAF and PIK3CA genes in the 67 xenograft models used as controls in the treatment experiments with cetuximab, bevacizumab and oxaliplatin*

A comparison of the mutation profile of the total set of 133 xenograft models (KRAS 34%; BRAF 13%; PIK3CA 13%) and the subset of 67 xenograft models (KRAS 49%; BRAF 10%; PIK3CA 10%) showed no significant differences in the mutation frequency in all three genes between the two groups (p=0.1970, OR: 0.6744 95% CI: 0.3701 - 1.229). If only BRAF and PIK3CA mutations were considered (p=0.5930, OR: 0.7755, 95% CI: 0.3046 - 1.974), no significant difference was observed between the two sets. If one compares the mutation rate in the KRAS gene between the two groups the difference is, however, statistically significant (p=0.0271, OR: 1.964, 95% CI: 1.075 - 3.587). The higher mutation rate was seen in the 67 models only in codon 12 (p=0.0698), and not in codon 13 (p=0.7585) or codon 146 (p=0.9716). In the 67 models used in the treatment experiments the mutation status of codon 61 of the KRAS gene was also investigated and two additional mutations were identified. The two mutations detected in codon 61 led to an overall higher frequency of KRAS mutations in the subset of the 67 xenografts. If one compares the frequency of KRAS mutations in the 67 xenograft models with the frequency of KRAS mutations in the clinical tumour samples of the MSKK/RVS study, the difference does also not achieve statistical significance for all KRAS mutations (p=0.3454), nor for mutations in codon 12 in particular (0.4678).

Tab.3.9. *Mutation distribution and response to cetuximab in a group of 67 xenograft models in respect to the baseline patient characteristics*

Feature	Total	BRAF	%	KRAS	%	PIK3CA	%	Double	%	Resp.p	%
Total n pts	67	7	10	33	49	7	10	6	9%	18	27
Gender											
Male	31	2	6	14	45	3	10	3	10	9	29
Female	36	5	14	19	53	4	11	3	8%	9	25
Age											
≤60 yrs old	15	0	-	7	47	2	13	2	13	4	27
>60 yrs old	52	7	13	26	50	5	10	4	8%	14	27
Location											
Colon	46	7	15	20	43	7	15	6	13	10	22
Rectum	21	0	-	13	62	0	0%	0	0%	8	38
UICC Stage											
I	8	1	13	5	63	1	13	1	13	2	25
II	22	2	9	7	32	1	5%	1	5%	8	36
III	28	3	11	18	64	5	18	4	14	4	14
IV	9	1	11	3	33	0	0%	0	0%	4	44

Among 67 treated models additional mutation analysis was performed in the two hotspots of CTNNβ1, the most commonly mutated position in the APC and five hotspots of TP53. TP53 point mutations were found in 11 (16%) of the 67 models, out of which six harboured additional mutation in KRAS, PIK3CA or BRAF. One mutation was detected in the CTNNβ1 gene (S45F) in the model carrying as well KRAS (A146T) and PIK3CA (H1047R) mutations. No R1450* mutations were observed in the APC gene in the investigated models.

3.4.3 Mutation Status of the 67 Xenograft Models and Response to Cetuximab

Mutations of KRAS, BRAF and PIK3CA were already linked with the resistance in the patients treated with anti-EGFR mAbs in the large clinical trials (Tab.S12). The mutation status of these genes was therefore analyzed in the context of the response to cetuximab among the 67 xenograft models. Of the 18 cetuximab responders, 15 responders are wild-type in all analyzed hotspots of the three genes (Tab.3.10). The remaining three responders carry mutations in the KRAS gene. Two models (M57 and M95) carry mutations in codon 13 (Fig.3.6 A and B) and one model (M128) carries a 34 G>A mutation in codon 12 (Fig.3.6 C).

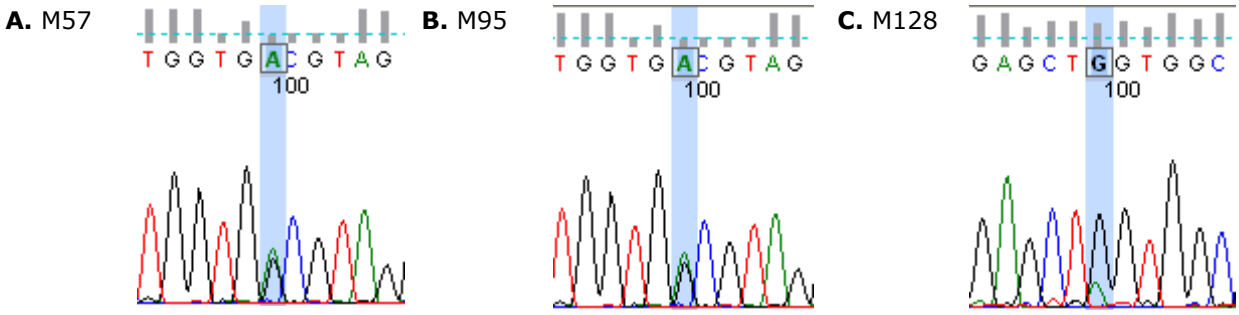


Fig.3.6. *Three xenograft models used in the treatment experiment that were classified as cetuximab responders and carry KRAS mutations in codon 13 (M57 - A; M95- B) or in codon 12 (M128 - C)*

In the non-responder group mutations KRAS codon 12 and 13 gene were found in 26 out of 49 of the cases. Additional KRAS mutations in codon 61 in codon 146 were found in 4 resistant cases. All seven mutations found in BRAF (V600E) were carried by cetuximab non-responders. Similarly, all seven PIK3CA mutations were found in the non-responding models. There was one CTNNB1 (S45F) mutation found in the model, which was resistant to cetuximab. Out of eleven TP53 mutations, detected in the group of the 67 xenografts, four were found among responders (4/18) and seven among non-responders (7/49).

If we apply a standard patient selection procedure, which considers mutations in codon 12 and 13 to cause resistance to anti-EGFR treatment, 15 out of 18 responders are wildtype and would be identified as responder to cetuximab treatment. This corresponds to sensitivity (S^+) of 83%. In the non-responder group mutations in KRAS codon 12 and 13 gene were found in 26 out of 49 of the cases resulting in a specificity of 53%. If we consider a more sophisticated selection procedure and assume that besides the standard KRAS mutations in codon 12 and 13 also KRAS mutations in codon 61 and 146 and mutations in BRAF and PIK3CA cause resistance to anti EGFR treatment, the specificity increases to 78% as 12 additional resistant cases can be recognized, while the sensitivity remains unchanged (83%).

Results of a recent study (De Roock et al., 2010b) suggest that not all mutations in codon 13 of KRAS are associated with resistance to cetuximab. In our data set, we found six 38 G>A mutations in codon 13. Two of them were identified in responders and four in non-responders. If we link KRAS codon 13 mutations with response to EGFR treatment and all other mutations with resistance, a predictor model can be built that is based on the combination of wild-type KRAS, BRAF, PIK3CA and KRAS codon 13 mutations. Such a predictor achieves an improved sensitivity of 94% (17/18 responders correctly recognized). However, its specificity would decrease to 69% as only 34 of the 53 resistant cases are properly classified.

In the subset of the 67 models we investigated additional hotspots in the CTNNß1, APC and TP53 genes which are frequently mutated in the CRC patients in order to increase the specificity of our predictor model. None of the mutations were linked to response or resistance to cetuximab.

Tab.3.10. *Mutation status of the analyzed hotspots (KRAS, BRAF, PIK3CA, CTNNB1, TP53 and APC) and T/C ratio of response (Resp.) toward cetuximab, bevacizumab and oxaliplatin of each of the 67 CRC xenograft samples used in the treatment experiment; T/C - volume of the treated tumour in relation to the non-treated control; responders were defined with a T/C ratio of <20%. T/C. *Rating: If T/C: > 50 %: "-", 36-50 %: "+", 21-35%: "++", 6-20 %: "+++", < 5 %: "++++"; NA - not applicable in some of the analyzed hotspots; UICC - Cancer Staging according to the Union for International Cancer Control (UICC; see Tab. S.2)*

Models /patients			Response to the treatment						Mutation status						Mut	UICC
			Oxaliplatin		Cetuximab		Bevacizumab									
#	model-ID	patient-ID	T/C	Rated	T/C	Rated	T/C	Rated	KRAS	BRAF	PIK3CA	CTNNB1	TP53	APC	No	stage
1	M128	-	15,9	+++	1,2	++++	20,3	++	34 G>A	WT	WT	WT	WT	WT	1	IV
2	M76	-	68,3	-	1,7	++++	41,0	+	WT	WT	WT	WT	WT	WT	0	II
3	M88	P812	69,3	-	2,7	++++	102,7	-	WT	WT	WT	WT	WT	WT	0	II
4	M122	-	66,3	-	3,2	++++	21,1	++	WT	WT	WT	WT	WT	WT	0	II
5	M82	P792	61,8	-	3,6	++++	80	-	WT	WT	WT	WT	WT	WT	0	II
6	M1	P494	40,9	+	4,5	++++	46,7	+	WT	WT	WT	WT	524 G>A	WT	1	II
7	M60	P704	35,5	++	5,3	+++	47,4	+	WT	WT	WT	WT	WT	WT	0	III
8	M112	-	116,7	-	5,3	+++	63,2	-	WT	WT	WT	WT	524 G>A	WT	1	IV
9	M93	P821	19,4	+++	7	+++	19,4	+++	WT	WT	WT	WT	743 G>A, NA	WT	1	III
10	M13	-	19,7	+++	7,6	+++	39,4	+	WT	WT	WT	WT	WT	WT	0	II
11	M98	-	37,7	+	8,6	+++	20,3	++	WT	WT	WT	WT	WT	WT	0	III
12	M29	-	68,4	-	8,9	+++	31,1	++	WT	WT	WT	WT	WT	WT	0	I
13	M79	P784	40	+	12	+++	38,7	+	WT	WT	WT	WT	WT	WT	0	IV
14	M99	-	37,7	+	13,1	+++	41,4	+	WT	WT	WT	WT	NA	WT	0	II
15	M84	-	65,5	-	17,2	+++	43,1	+	WT	WT	WT	WT	WT	WT	0	IV
16	M95	-	86,4	-	17,3	+++	34,9	++	38 G>A	WT	WT	WT	WT	WT	1	I
17	M124	-	61,0	-	17,5	+++	43,9	+	WT	WT	WT	WT	WT	WT	0	II
18	M57	P698	84,3	-	19,6	+++	20,6	++	38 G>A	WT	WT	WT	743 G>A, NA	WT	2	III
19	M47	-	22,2	++	22	++	25,9	++	35 G>C	WT	WT	NA	WT	WT	1	II
20	M52	-	51,5	+	24,8	++	64,6	-	35 G>C	WT	WT	WT	WT	WT	1	III

#	model-ID	patient-ID	T/C	Rated	T/C	Rated	T/C	Rated	KRAS	BRAF	PIK3CA	CTNNB1	TP53	APC	No	Stage
21	M85	-	62,2	-	27	++	54,5	-	35 G>A	WT	WT	WT	WT	WT	1	II
22	M114	-	22,5	++	27,1	++	35,4	++	35 G>C	WT	WT	WT	NA	WT	1	II
23	M102	-	4,7	++++	28	++	24,3	++	35 G>T	WT	WT	WT	WT	WT	1	III
24	M83	P801	58,1	-	32	++	26,7	++	35 G>C	WT	WT	WT	WT	WT	1	III
25	M117	-	72,2	-	33,8	++	48,7	+	WT	WT	1633 G>A	WT	NA	WT	1	III
26	M91	-	186,2	-	35,3	++	53	-	WT	WT	WT	WT	WT	WT	0	II
27	M27	P596	42,2	+	36,9	+	16,5	+++	35 G>A	WT	WT	WT	818 G>A	WT	2	III
28	M72	-	43,9	+	38,1	+	40,4	+	WT	WT	WT	WT	WT	WT	0	II
29	M75	P775	56,6	-	39,6	+	50,9	-	35 G>C	WT	WT	WT	WT	WT	1	III
30	M53	-	25,4	++	40,8	+	36,6	+	35 G>A	WT	WT	WT	WT	WT	1	IV
31	M80	-	76,6	-	42,4	+	90	-	WT	WT	WT	WT	WT	WT	0	II
32	M107	-	57,1	-	42,9	+	22,2	++	38 G>A	WT	WT	WT	WT	WT	1	III
33	M23	-	56,7	-	43,7	+	24,5	++	WT	WT	WT	WT	WT	WT	0	IV
34	M87	P808	52,2	-	45,6	+	108,7	-	WT	WT	WT	WT	WT	WT	0	IV
35	M94	-	45,2	+	46,7	+	31	++	WT	WT	WT	WT	WT	WT	0	II
36	M129	-	28,6	++	47,7	+	52,4	-	35 G>T	WT	WT	WT	WT	WT	1	IV
37	M66	P712	45,7	+	48,6	+	41,2	+	183 A>T	WT	WT	WT	NA	WT	1	II
38	M132	-	22,3	++	49,2	+	21,4	++	436 G>A	WT	3140 A>G	134 C>T	WT	WT	3	I
39	M118	-	61,5	-	51,2	-	58,2	-	35 G>T	WT	3140 A>G	WT	524 G>A	WT	3	III
40	M104	-	67,7	-	51,6	-	47,4	+	38 G>A	WT	WT	WT	WT	WT	1	III
41	M106	-	31,5	++	53,2	-	64,3	-	35 G>T	WT	WT	WT	WT	WT	1	I
42	M68	P733	68,4	-	55,2	-	57,9	-	35 G>T	WT	WT	WT	NA	WT	1	III
43	M55	P692	58,3	-	58,3	-	33,3	++	WT	WT	WT	WT	844 C>T	WT	1	III
44	M81	P790	37,0	+	60,9	-	57,2	-	WT	1799 T>A	WT	WT	WT	WT	1	III
45	M61	P708	93	-	61	-	88,2	-	35 G>T	WT	WT	NA	WT	WT	1	I
46	M86	-	53,6	-	62,5	-	50	+	35 G>A	WT	WT	WT	WT	WT	1	III
47	M89	-	30,2	++	62,5	-	87,5	-	WT	WT	WT	WT	NA	WT	0	III
48	M123	-	38,8	+	63,4	-	38,4	+	WT	1799 T>A	WT	WT	WT	WT	1	III

#	model-ID	patient-ID	T/C	Rated	T/C	Rated	T/C	Rated	KRAS	BRAF	PIK3CA	CTNNB1	TP53	APC	No	Stage
49	M77	-	37,0	+	64,4	-	38	+	WT	WT	WT	WT	818 G>A	WT	1	II
50	M101	-	60,9	-	65,7	-	50	+	34 G>A/T	WT	WT	WT	WT	WT	1	III
51	M18	P574	72,9	-	67,8	-	103,4	-	35 G>A	WT	WT	WT	WT	WT	1	II
52	M121	-	43,2	+	69,3	-	31,8	++	WT	1799 T>A	WT	WT	WT	WT	1	IV
53	M120	-	48,5	+	69,7	-	54,6	-	WT	1799 T>A	WT	WT	WT	WT	1	II
54	M43	P662	61,5	-	72,7	-	66,6	-	WT	1799 T>A	WT	WT	WT	WT	1	I
55	M105	-	90	-	73,1	-	56	-	WT	WT	WT	WT	WT	WT	0	III
56	M92	-	71,4	-	74,7	-	88,7	-	35 G>T	WT	WT	WT	WT	WT	1	III
57	M110	-	83,3	-	79,2	-	67,8	-	182 A>T	WT	1624 G>A	WT	WT	WT	2	II
58	M63	P710	34,7	++	79,2	-	23,6	++	38 G>A	WT	WT	WT	WT	WT	1	III
59	M90	-	71	-	79,3	-	82,8	-	35 G>T	WT	1633 G>A	WT	WT	WT	2	III
60	M59	P702	95,3	-	81	-	54,2	-	WT	1799 T>A	WT	WT	742 C>T, NA	WT	2	III
61	M65	-	85,9	-	81,4	-	37,2	+	436 G>A	WT	1633 G>A	WT	743 G>A, NA	WT	3	III
62	M115	-	77,3	-	82,1	-	54,6	-	38 G>A	WT	WT	WT	WT	WT	1	II
63	M56	P693	75	-	88	-	66,7	-	35 G>T	WT	WT	WT	WT	WT	1	I
64	M97	-	52,2	-	89,7	-	53,6	-	WT	WT	WT	WT	WT	WT	0	I
65	M125	-	88,2	-	93	-	34,5	++	35 G>T	WT	WT	WT	WT	WT	1	III
66	M33	P621	54,7	-	94,7	-	63,8	-	WT	1799 T>A	WT	WT	743 G>A, NA	WT	2	II
67	M96	-	90,3	-	120	-	70,3	-	35 G>T	WT	1633 G>A	WT	WT	WT	2	III

3.4.4 RNA Expression and Response to Cetuximab

In addition to mutation markers, RNA expression markers were investigated using TaqMan real-time PCR. AREG, EREG and PTEN expression levels were analyzed in the RNA isolated from the first set of passages of the 67 treated xenograft models. The normalized Ct values are given in the supplementary table S8. A significant correlation was found between high expression of AREG and EREG (low Ct values) and response to cetuximab. The Pearson Correlation Coefficient (PCC) was calculated between the normalized expression values and the T/C ratio, as an indicator of tumour response. PCC values indicating association with response to cetuximab were: 0.65 and 0.63 for AREG and EREG respectively. PTEN expression was not correlated with cetuximab response (PCC=0.06). Correlation calculated for AREG and EREG was even stronger in the group of xenograft models with KRAS wild-type (AREG: PCC=0.72; EREG: PCC= 0.75), while the PCC value for PTEN (PCC=0.07) did not increase significantly. For 67 cases a PCC greater than 0.2 (20%) is significant. DUSP6 and SLC26A3 two candidate genes were additionally analyzed in this setting and a correlation between their expression and the T/C values was calculated. For SLC26A3 a PCC of 0.38 was obtained and for DUSP6 an inverse correlation of PCC=-0.19. Among KRAS wild-type samples SLC26A3 reached a PCC value of 0.57 and DUSP6 of -0.36.

3.4.5 Receiver Operating Characteristic (ROC) Curves of Different Biomarkers

To verify and compare the accuracy of the classifiers ROC curves were constructed based on the assumptions that the following factors are predictive for resistance to cetuximab: 1. KRAS mutation codon 12 and codon 13 (dashed red); 2. any mutation in KRAS, BRAF and PIK3CA (dashed green); 3. any mutation in KRAS, BRAF and PIK3CA without KRAS codon 13 mutations (dashed brown); and finally 4. any mutation in KRAS, BRAF and PIK3CA without KRAS coding 13 mutations and mean AREG and EREG RNA expression (blue; Fig.3.7).

The area under the curve (AUC) for the four different scenarios is: 0.68, 0.80, 0.82, and 0.96 respectively. Tables that served for the construction of the ROC curves and that display the potential of the expression values of analyzed genes to recognize responders/non-responders are shown in Tab.S9.

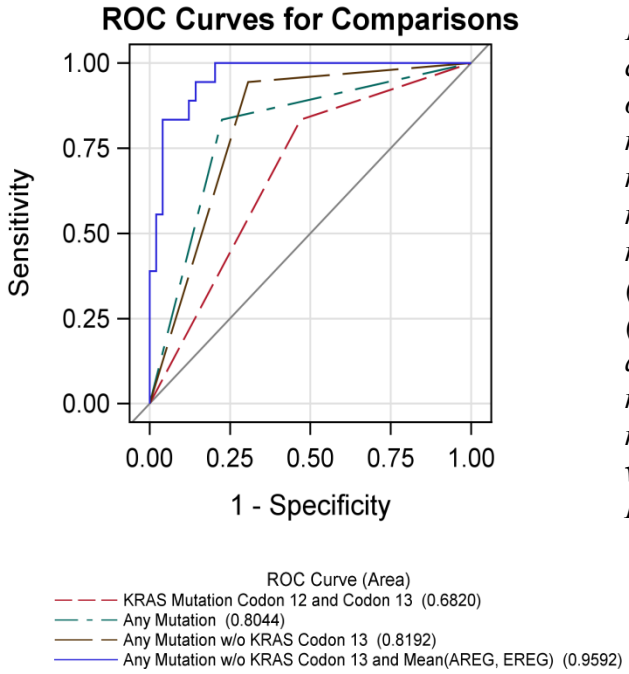


Fig.3.7. *Receiver operating characteristic (ROC) curve for prediction of patient response to cetuximab as measured in the panel of 67 xenograft models by: 1:KRAS codon 12 and 13 mutations (dashed red); 2: all analyzed mutations in KRAS, BRAF, and PIK3CA (dashed green); 3: by mutations in KRAS (excluding codon 13 mutations), BRAF and PIK3CA (dashed brown); 4: by mutations in KRAS (excluding codon 13 mutations), BRAF and PIK3CA combined with RNA expression of AREG and EREG (blue) in the set of 67 xenograft models)*

3.4.6 anti-EGFR Response Prediction by GEP on the U133 Plus 2.0 Arrays

Encouraged by the correlation observed between the RNA expression level of selected genes and response to anti-EGFR mAbs I decided to apply as well genome-wide gene expression profiling on the Affymetrix array platform. Affymetrix U133 Plus 2.0 array analysis was carried out for 66 out of 67 models that were used in the treatment experiment. RNA samples were isolated from the tumour tissues of the non-treated xenograft controls. There were five non-treated controls for each model; therefore RNA was pooled after completing individual RNA extractions, prior to the hybridization on the array. Altogether 18 responders and 48 non-responders were analyzed. First, the raw CEL files were normalized using the FARMS program. FARMS condensation identified 14753 informative probesets out of the 54.000 probesets contained on the U133 2.0 Plus array. Then a nested bootstrap method with random forest was applied to the 14753 probesets for all 66 samples. A signature, which contains 1.800 probesets classified responders and non-responders with the highest prospective sensitivity of 89.7% and a prospective specificity of 78.1% (Fig.3.8). A short signature of 54 probesets had a sensitivity of 85.2% and a specificity of 81.1%.

A signature obtained in such manner contains probesets derived from genes coding for proteins of known function but also from genes coding for hypothetical proteins. The list of 100 best performing probesets and related S+/S- is displayed in the supplementary table S10. The first twenty predictive probesets corresponding to 18 genes are listed also in Tab.3.11.

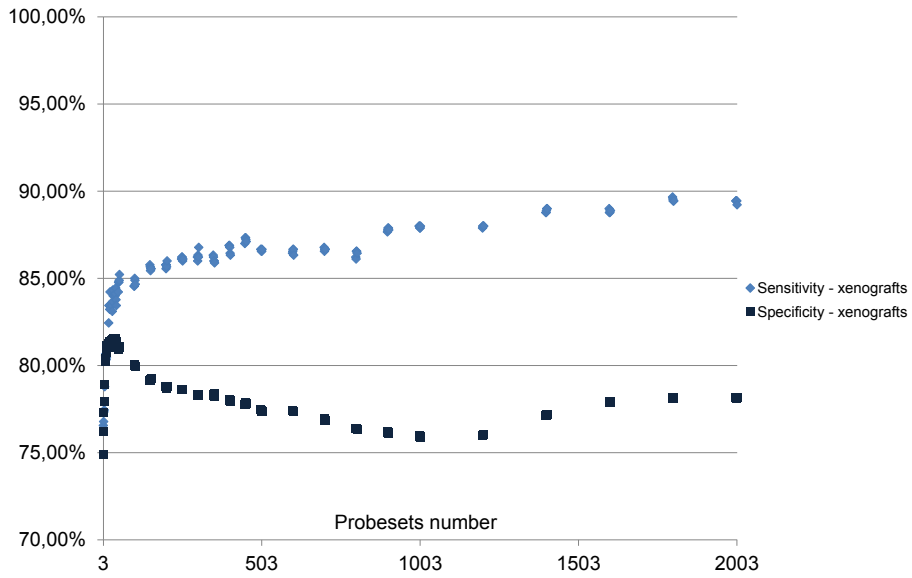


Fig.3.8. *Prospective performance of the mRNA signatures during balanced discovery on the panel of xenografts (18 responders (R) vs. 48 non-responders (NR)); matched primary tumours (18 R vs. 43 NR). The plot shows the changes in sensitivity and specificity according to the number of best performing probesets included in the classifier*

The probeset with the highest rank belongs to a gene (hypothetical protein LOC158960) of unknown function. Among the first twenty probesets are, however, many that were previously linked to tumorigenesis and neoplastic processes. The second best classifying gene is epiregulin, a ligand of EGFR. Another important ligand of EGFR, amphiregulin B, is ranked on the 7th place. The function of the arginine-glutamic acid dipeptide repeats (RERE) gene that is ranked on the 3rd place is to repress transcription and to trigger apoptosis. The protein tyrosine phosphatase (PTPRF) which plays a role in the Wnt signaling pathway appears twice among the twenty best classifying probesets. B-cell translocation gene 1 (BTG1), ranked on the 8th place, is a gene which belongs to the anti-proliferative gene family that regulates cell growth and differentiation. An inhibitor of TNF-induced apoptosis Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1) is listed on place 13. A serine/threonine kinase, which interacts with SMAD transcription factors and is involved in the MAPK and TGF-beta signaling pathways, was identified on the position 18. Right afterwards another gene that plays a role in signal transduction - GNB1 appears in the classification. There were also probesets

corresponding to the genes involved in the protein sorting in endoplasmic reticulum (KDELR2), regulating membrane traffic to and from trans-Golgi network (TGOLN2) or glycosylation in the Golgi apparatus (B3GNT7). Other genes listed among the 20 best classifiers are RecQ DNA helicase (RECQL4), protein dpy-19 homolog 1 (DPY19L1) and a possible splicing regulator involved in the control of cellular survival (SFRS12IP1). The probesets listed on the position 4, 16 and 17 detect genes coding uncharacterized proteins.

Tab.3.11. *A list of probesets scoring the 20 best results according to the importance in the discrimination between responders and non-responders to cetuximab according to the discovery in xenograft models and primary tumours*

	Affymetrix probeset	Gene symbol	Description
1	1558685_a_at	LOC158960	hypothetical protein BC009467
2	1569583_at	EREG	epiregulin
3	200940_s_at	RERE	arginine-glutamic acid dipeptide (RE) repeats
4	1568597_at	LOC646762	hypothetical LOC646762
5	1553581_s_at	SFRS12IP1	SFRS12-interacting protein 1
6	200636_s_at	PTPRF	protein tyrosine phosphatase
7	1557285_at	AREGB	amphiregulin B
8	200920_s_at	BTG1	B-cell translocation gene 1
9	200698_at	KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2
10	1555962_at	B3GNT7	UDP-GlcNAc:betaGal beta-1
11	1553015_a_at	RECQL4	RecQ protein-like 4
12	200921_s_at	BTG1	B-cell translocation gene 1
13	200976_s_at	TAX1BP1	Tax1 (human T-cell leukemia virus type I) binding protein 1
14	1554608_at	TGOLN2	trans-golgi network protein 2
15	1560916_a_at	DPY19L1	dpy-19-like 1 (C. elegans)
16	1558412_at	LOC113230	hypothetical protein LOC113230
17	1559957_a_at	LOC642852	hypothetical LOC642852
18	1552519_at	ACVR1C	activin A receptor
19	200745_s_at	GNB1	guanine nucleotide binding protein (G protein)
20	200637_s_at	PTPRF	protein tyrosine phosphatase

3.4.7 anti-EGFR Response Prediction and microRNA Expression

Also microRNA expression signatures were tested in relation to response to cetuximab. Total RNA isolated from the tumour tissue of the non-treated controls corresponding to the subset of 18 responding xenografts and 19 resistant xenografts was pooled and hybridized onto Affymetrix miRNA 2.0. This arrays cover all the miRNAs reported in the miRBase V15 (http://www.mirbase.org/) of 131 organisms. Analysis of the best discriminating probesets revealed that the signatures of significant predictive benefit required at least 100 probesets (sensitivity: 66.7%; specificity: 66.5%; see Fig.3.9). A classifier, which contains 250 probesets, performed with a sensitivity of 73% and with a

specificity of 67.6%. Accuracy increased with a number of probesets and reached 77.1%(S+)/66.4%(S-) and 80.7%(S+)/63.6%(S-) for a signature containing 500 and 1000 probesets respectively. A signature including all 1404 informative probesets had the highest sensitivity of 82.3% and a specificity of 73.1%. The list of 100 best performing probesets is displayed in the supplementary Tab.S11. Altogether, there were probesets corresponding to the genes of 50 different organisms. Interestingly, the probesets of human miRNA were rarely the best scoring probesets. Thus, a potential signature that contained only human miRNA sequences performed worse than a signature which contained miRNA probesets from human and other species.

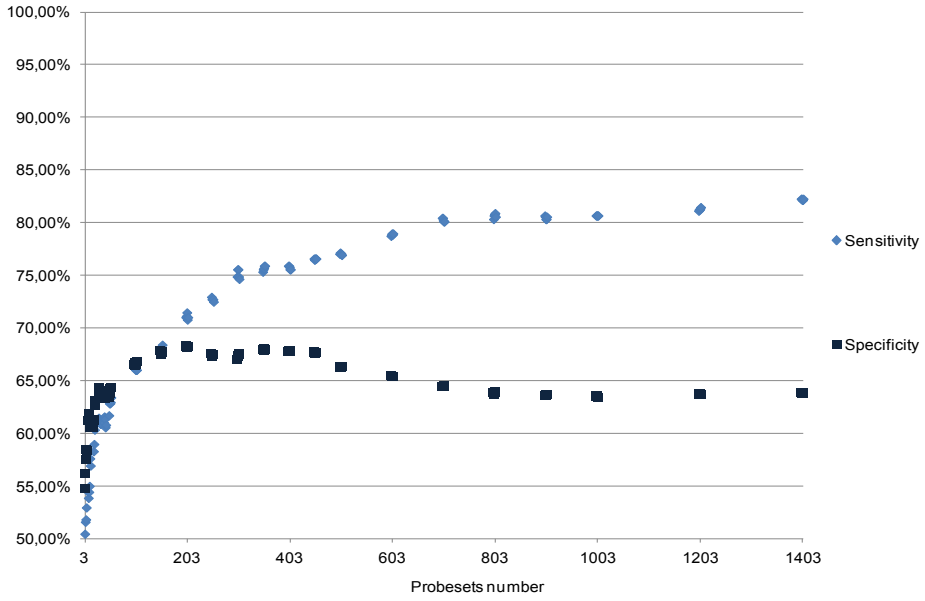


Fig.3.9. *Prospective performance of the miRNA signatures during balanced discovery on the panel of xenografts (18 responders (R) vs. 19 non-responders (NR)). Plot shows the changes in sensitivity and specificity according to the number of best performing probesets included in the classifier*

Chapter 4

Discussion

4.1. UNMET MEDICAL AND DIAGNOSTIC NEED IN COLORECTAL CANCER

In spite of the many advances in early detection, surgical techniques, therapeutics and molecular characterization made over the past decade colorectal cancer remains still a major health problem. It is the third most common cancer in man and the second most common cancer in women and one of the most aggressive and deadly solid cancers (Jemal et al., 2011) with a high, unmet clinical need.

Improved strategies and new treatment options were developed for colorectal cancer patients across different stages of the disease, primarly for patients with advanced metastatic colorectal cancer. Although three targeted antibodies including cetuximab, panitumumab and bevacizumab were approved since 2004, the 5-year overall survival of patients with mCRC remained unchanged and is still dramatically low (4-12%). Why targeted therapies do not have a higher impact on survival in mCRC patients?

One of the reasons for their poor performance is an ability of the tumours to bypass the first generation targeted cancer drugs by use of different signaling cascades. Often, after

strong initial response, tumours escape the mechanism of action of the therapeutic agent and develop resistance. The different escape and resistance mechanisms remain poorly understood in most of the cases (Misale et al., 2012). Solid tumours have a high degree of intra-heterogeneity and growth signals can be transferred through different signaling pathways, therefore targeting a single receptor or a single ligand seems to be insufficient. Moreover, the tumour epithelium interacts in a complex way with different surrounding cell types also described as tumor microenvironment (TME). These interactions are still poorly understood. In addition, tumour epithelial cells have the ability to acquire resistance under the selective pressure of cancer drugs (Diaz, Jr. et al., 2012).

A second reason for the limited efficacy of targeted therapies is that only a small fraction of patients benefit from these drugs. Thus stratification of patients should be carefully conducted prior to the treatment. However, responders/resistant cases cannot be efficiently preselected as accurate biomarkers are not yet recognized. KRAS mutation testing has been established and is mandatory prior to the treatment with cetuximab and panitumumab. It is assumed that wildtype KRAS patients benefit from these two anti EGFR antibodies while patients with KRAS mutations do not. However, careful analysis of tumour samples of patients treated with these two drugs showed that not all KRAS wild-type patients benefit from the targeted anti-EGFR antibodies. Thus, KRAS alone is not an accurate predictive biomarker. For bevacizumab, a mAb directed at the VEGF-A ligand, no biomarkers have been identified so far.

A significant obstacle to the search for predictive biomarkers in patients with mCRC is the availability of tumour tissue. Metastasis tissue is accessible prior to treatment in a small fraction of patients. For the majority of mCRC patients only primary tumour tissue is available for biomarker testing. Primary tumour tissue, however, does not reflect the molecular changes which liver or lung metastases have acquired or potential changes induced by the drug itself through evolutionary pressure. For majority of the mCRC patients who receive neoadjuvant treatment, no current primary tumour tissue is available for biomarker testing.

Preclinical models have been therefore established to provide a tool for an extensive and systematic testing of novel drugs. Cancer cell lines and the NCI60 panel in particular have been used in many in-vitro studies (Suggitt and Bibby, 2005). Due to the disadvantages of the in-vitro approach, such as the limited number of the cancer cell lines and their specific properties gained during decades-long cultivation in the laboratory, several studies investigated also cancer xenograft models. In most of the

cases however, cancer cell lines and not the actual tumour tissues were transplanted on the animal (Balin-Gauthier et al., 2006a; Luo et al., 2005b; Prewett et al., 2007a). Nevertheless, neither cancer cell lines nor xenograft models derived thereof, reflect the heterogeneity and complexity of the tumour (Caponigro and Sellers 2011) and the variety of the patients.

There are also regulatory hurdles in the development process. Current clinical trial regulations do not allow testing of novel targeted drugs in chemonaive patients. Novel cancer drugs need to be assessed in 3rd or 4th line in heavily pre-treated mCRC patients. These patients have already been exposed to adjuvant therapy or have undergone 1st line and 2nd line therapy with FOLFOX or FOLFIRI regimens alone or in combination with targeted agents. Most of them already showed resistance to the treatment and have often only weeks to live, therefore to prove efficacy of a drug and survival benefit in such group of patients, is very difficult.

As we cannot change these clinical regulations, there is a need to develop novel preclinical strategies, which allow testing of investigational compounds and establishing biomarkers early in the drug discovery process. In this Ph.D. thesis I evaluated a new preclinical strategy in which a significant number of xenograft models derived from primary colorectal cancer tumour specimens was treated with three drugs approved in colorectal cancer: cetuximab, bevacizumab and oxaliplatin. Tumour tissue of the xenografts was then used for the molecular characterization on the genetic and epigenetic level. Response/resistance data obtained in the treatment experiment with xenografts were correlated with the molecular features in order to identify predictive biomarkers for drug response to the therapy.

4.2. MSKK/RVS Clinical Trials as a Representation of the CRC Patients' Population and an Origin of the Engrafted Samples

4.2.1. Clinicopathological features of the analyzed subset of CRC population

In order to test whether the sample collection exhibits a random representation of the CRC population of all four stages extensive molecular and statistical analyses were performed in the subgroup of the 960 patients' samples originating from the tumour tissue bank. Altogether 96 clinical samples were excluded from the original subgroup of 960 patients due to clinical, pathological and ethical criteria, leaving a cohort of 864 patients available for further analyses. Basic clinical and pathological characteristics of the 864 patients and tumour samples were compared with the tumour register of the state Brandenburg, particularly with patients diagnosed with colorectal cancer in the state Brandenburg between 2000 and 2009 (http://www.tumorzentrum-

brandenburg.de). The comparison of the patients' features suggests that the samples analyzed in our study constitute a random fraction of the colorectal cancer patient population and its balanced representation.

4.2.2. Mutation distribution in the analyzed subset of CRC population

Analysis of the mutation distribution in KRAS, BRAF and PIK3CA was conducted by allele-specific real-time PCR in the group of 864 patients. This approach allowed analyzing nine different hotspot mutations in KRAS, the most frequent mutation in BRAF and three mutations in PIK3CA. In 53% of patients at least one mutation in the analyzed hotspots of KRAS, BRAF and PIK3CA was found. Next, the frequencies of selected mutations were compared with the frequencies reported in the COSMIC database (Tab.3.4). The distribution of the mutations in the three genes KRAS, BRAF and PIK3CA is in agreement with the COSMIC database in most cases.

A substantial difference in the frequency between our results and the COSMIC database was observed in one of the nine analyzed hotspots in the KRAS gene: codon A146T. COSMIC displays the A146T at a frequency of 0.1% only, while in the MSKK/RVS study this mutation was found in 2.2% of the samples. A reason for this difference may be the structure of the COSMIC that screens the information available on the somatic mutations in cancer in the published scientific literature and displays it in relation to particular human cancers, their morphology, histology or mutated hotspots. Such design of data collection leads to a systematic bias. Codons 12 and 13 of the KRAS gene are in the focus since these hotspots were associated with resistance to cetuximab in 2006, while other KRAS mutations were analyzed less commonly. An oncogenic effect of mutations in codon 146 of KRAS was reported later and their influence on the response to the anti-EGFR monoclonal antibodies has not yet been evaluated in a large clinical trial (Loupakis et al., 2009). As a consequence, KRAS codon 146 has been studied less frequently and its frequency in the COSMIC database is biased as compared with other hotspots. The frequency of the A146T mutation of 2.2% as observed in our setting is, however, in agreement with findings of several other studies (Edkins et al., 2006; Loupakis et al., 2009; Vaughn et al., 2011).

Also mutations in the PIK3CA gene, E545K in particular, were reported with higher frequency among the MSKK/RVS population than in the COSMIC database. A substitution of G to A in the nucleotide position 1633 in exon 9 of PIK3CA results in the amino acid change E545K and was detected in almost 8% of analyzed patients. These finding are, however, in concordance with a population based study conducted in 743 patients (De Roock et al., 2010a).

There was no significant difference observed in the frequency of V600E BRAF mutation between our study (9.5%) and the COSMIC database (10.1%). BRAF mutations were also mutually exclusive with KRAS mutations ($p \leq 0.0001$) (except for two patients) which confirms the previous observations (Rajagopalan et al., 2002).

4.2.3. Correlation of mutations in KRAS, BRAF and PIK3CA with the clinicopathological data of the 864 patients

A statistical analysis of the clinicopathological patient data and the corresponding mutation status was performed using the Chi-square test. The results indicated that KRAS mutations are not correlated to gender, age, tumour location, or the UICC-stage ($p>0.1$). This observation stays in agreement with most of the published population-based studies. In contrast, BRAF mutations are linked to the female gender and tumour location in colon. There was a trend in the correlation of BRAF mutation and more advanced UICC stage ($p= 0.0632$). Our results support previous reports that BRAF mutation status is correlated with specific clinicopathological features and therefore identifies a subgroup of colorectal cancers with distinctive clinical and pathological characteristics (Li et al., 2006; Zlobec et al., 2010). A weak association of PIK3CA mutations and tumour localization in colon, observed in our analysis, was not found in other studies (Sartore-Bianchi et al., 2009b; Naguib et al., 2011). The 864 MSKK/RVS colorectal cancer samples seem to be in agreement with other population-based studies, not only in terms of the distribution of mutations or clinical and pathological features, but also in respect to the associations between mutation status and clinical data.

4.3. SENSITIVITY OF THE ALLELE-SPECIFIC REAL-TIME PCR FOR MUTATION DETECTION

To test the sensitivity of each mutation assay, I performed titrations of mutated DNA isolated from the tumour cell lines with wild-type genomic DNA. Most of the allele-specific real-time PCR assays detected mutations with a sensitivity of 2.5-5% mutated allele content. Some assays, however, had a sensitivity of 10%. A general cut-off at 10% was set according to the assays with the lowest sensitivity. Performance of the allele-specific real-time PCR method was additionally compared with direct sequencing in KRAS codon 12 and 13 in a subset of 304 out of 864 samples (35%). In 98% of the patients the results are identical. In five cases the mutation was detected only by allele-specific real-time PCR assays but not by conventional sequencing suggesting that the allele-specific PCR assays are more robust in detecting KRAS mutations in tumour samples with lower content of mutant allele. Moreover, the short PCR fragments of 80-90 bp used in the allele-specific method allow analyses of the samples of poor quality. Such short PCR products are not optimal in dideoxy-sequencing and the 283 bp fragment used for

dideoxy sequencing led to the failure of 21 additional samples in this setting. Our results suggest superiority of the real-time PCR-based assays over direct dideoxy sequencing in terms of sensitivity.

Another research group (Kotoula et al., 2009) compared the allele-specific real-time PCR used in our study with a commercially available allele-specific test (TheraScreen® K-RAS Mutation Kit, DxS, Manchester, UK) and with conventional sequencing. One hundred thirty five diagnostic samples and 75 low-quality samples were analyzed. 109/111 samples, analyzed by both: allele-specific real-time PCR and sequencing, showed matching results. The two discrepant cases were determined as mutants by allele-specific real-time PCR and as wild-type by direct sequencing. The results of allele-specific real-time PCR and the commercial test conducted in the subset of 66 samples were in full concordance. In the subset of samples with low quality of DNA the cut-off for the real-time based methods was set at 10%, which is in concordance with the cut-off applied in our study. In comparison, the sensitivity of conventional sequencing was determined at the detection threshold of 30% of mutant alleles and could only be applied in the samples with relatively well preserved DNA. Furthermore, similar to our observation, in some cases more than one mutation was detected in the same tissue sample in KRAS codon 12 by allele-specific PCR as well as by the commercial test, suggesting cross-reactivity of the probes.

Our results, supported by the findings of other research groups, indicate that allele-specific real-time PCR assay is a reliable mutation detection method. Due to the high sensitivity and the use of short PCR fragments this method can be applied to tissue samples with low tumour cell content or to FFPE samples, which exhibit low quality of DNA. Allele-specific real-time PCR is more sensitive than the current standard of conventional sequencing. Moreover, if blocking oligonucleotides are added to the assays, this method has a high accuracy comparable to the performance of more expensive commercially available PCR methods.

4.4. THE PANEL OF XENOGRAFT MODELS AS A REPRESENTATION OF THE CRC PATIENTS

4.4.1. 133 Xenograft Models as a Representation of the CRC Patients' Population

The panel of 133 xenograft models derived from patients with colorectal cancer enrolled in the MSKK study. Therefore the clinical data of this cohort were investigated with respect to the general MSKK patient population. There is no difference in the basic clinical characteristics such as age, tumour location or gender in the two cohorts. The distribution of UICC stages II, III and IV in the 133 xenograft models is not statistically

different (p=0.14) to the stage distribution in a larger clinical cohort of 3.394 patients with colorectal cancer of stage II, III and IV that were recruited to the MSKK study. However, a statistical difference in UICC stage I was observed. UICC stage I comprise T1N0M0 and T2N0M0 tumours. Due to the fact that T1 tumours are very small, there are often not enough tumour specimens available for engraftment without compromising the histopathological diagnosis. Therefore, T1 tumors are underrepresented in the xenograft collection. We also compared the mutation distribution in the 133 xenograft models and the cohort of 864 patients of MSKK/RVS study and no significant difference was found in the overall mutation frequency in KRAS, BRAF, and PIK3CA.

Although the mutation rates in the 67 models used for the treatment experiments is in agreement with some of the previous studies (Baldus et al., 2010), a comparison with the panel of 133 xenograft models revealed that the prevalence of KRAS mutations, particularly those in codon 12, is higher among the 67 xenograft models used in the treatment experiment. A probable explanation for this bias is that the 133 xenograft models exhibit very different growth characteristics and that for the drug treatment experiments faster growing tumours were possibly selected. The growth rate of selected tumour models could be linked to their greater aggressiveness due to the presence of activating oncogenic mutations, indicating that mutations in KRAS codon 12 might be responsible for this process. Additional experimental verification is needed to confirm the association between different growth rates of some xenografts and particular oncogenic mutations. Compared to the overall patient population such difference in the mutation frequencies was not observed.

4.4.2 Xenograft Models as a Representation of Individual Patients' Tumours

Regarding the distribution of the mutations in KRAS, BRAF and PIK3CA and the basic clinical characteristics of the patients, the panel of 133 xenografts and its treated fraction of 67 xenografts reflect the population of CRC patients to a high degree. Subsequently, the histology of individual primary tumours resected from the patients and the tumour tissue of their matched xenograft models was compared. Histological examination of 133 samples revealed only eleven discrepancies between the original tumours and the corresponding xenograft models. Four of them, however, disappeared within the next set of passages. A difference may be a result of the heterogeneity of the original tumour, of which the xenograft contains only a part.

Overall, these results indicate that in the majority of the cases, the panel of 133 xenograft models preserved the histological properties of the original primary tumours.

Finally, the global RNA expression of the 133 xenograft tumours was compared with their matched primary tumour tissue using the Affymetrix U133 2.0 Plus array. Results of the microarray GEP approach show that the global RNA expression of the xenograft tumours is much closer to their respective matched human primary tumours than to other xenograft tissues (data not shown).

4.5. TREATMENT EXPERIMENTS WITH CETUXIMAB, BEVACIZUMAB AND OXALIPLATIN

Three drugs were selected to be tested in the same subgroup of 67 xenograft models: oxaliplatin as a standard chemotherapy regime and two mAb: bevacizumab and cetuximab. Panitumumab, another targeted mAb was not yet approved in CRC at the beginning of the project. Moreover panitumumab, similarly to cetuximab, targets the EGF receptor and was therefore not tested in the panel of xenografts. All three tested drugs were administered as single-agents to investigate their own cancer suppressive impact and to prevent overdoses.

The size of the treatment experiments was chosen in order to mimic clinical phase II studies, which often include 50-100 patients. Efficacy of the three drugs was tested in the same 67 xenograft models. Such setting for the comparison of different drugs cannot be achieved in clinical practice. Responders were prospectively defined with a T/C ratio of <20%. Following this strict criterion response was observed in 27% (18/67) treated with cetuximab. Bevacizumab resulted in the response rate of 3% (2/67) only and oxaliplatin in the response rate of 6% (4/67).

4.5.1 Oxaliplatin

Oxaliplatin is the only platinum agent currently approved in colorectal cancer. It is used commonly as a part of the FOLFOX or CAPOX regimens. When evaluated as single-agent with untreated CRC patients, oxaliplatin achieved response rates of 18-20% in a phase II study (Becouarn and Rougier, 1998; Diaz-Rubio et al., 1998), while in previously treated patients an objective response rate of 10% was reported in phase II study (Machover et al., 1996). However, the multicenter EFC 4584 trial, which led to the approval of oxaliplatin showed that the arm of the study that received oxaliplatin as a single-agent resulted in an overall response rate (ORR) of only 1.3% (Rothenberg et al., 2003). In our panel of chemonaive xenograft models a moderate response rate of 6% (95% CI: 2%-15%) was observed which is in between the published data.

4.5.2 Bevacizumab

Only 2 of the 67 (3%) xenograft models responded towards bevacizumab (95% CI: 1%-13%). Nine further xenograft models had a T/C ratio between 0.20 and 0.25

corresponding to a response rate of 11/67 (16%). Thus, with less stringent criteria the response rate to bevacizumab would be significantly higher.

In colorectal cancer bevacizumab is approved for use only in combination with 5-FU-based chemotherapy as a 1st or 2nd line treatment of metastatic colorectal cancer patients achieving a response rate of app. 20%. Single-agent bevacizumab used in a treatment of advanced disease is inferior to the combination with chemotherapy and shows limited efficacy of 3.3% (Cohen et al., 2007), which is comparable with our results of 3%. Interestingly, the two xenografts that responded to bevacizumab in our setting are derived of stage III patients, while recent results of a phase III clinical trial in patients with CRC of UICC stage II or III suggest no benefit from bevacizumab (Allegra et al., 2011).

4.5.3 Cetuximab

Out of three tested drugs only cetuximab showed benefit in a representative subset of 18/67 (27%) of the xenograft models. Cetuximab is approved in combination with chemotherapy (irinotecan or FOLFOX) as well as a single-agent in patients who have failed in oxaliplatin- or irinotecan- based chemotherapy. In the original BOND study, which led to its approval, a lower response rate for cetuximab monotherapy was observed in 11% of 329 treated patients (Cunningham et al. 2004). In this trial however, only irinotecan-refractory patients with metastatic disease were treated with cetuximab in third line, while in our study xenograft models derived of chemonaive patients of all four UICC stages were treated.

Since 2004 several clinical studies tested performance and efficacy of the anti-EGFR agents. A list of clinical trials with cetuximab and panitumumab is shown in supplementary table S12. Only a few trials investigated the efficacy of anti-EGFR agents as monotherapies and confirmed the results of the BOND study, achieving response rate of 8-11% for cetuximab (Giusti et al., 2007; Jonker et al., 2007; Karapetis et al., 2008a). In these trials only chemorefractory mCRC patients were enrolled. Recently, randomized trials that recruited only chemonaive patients were performed to investigate the benefit of adding cetuximab to either FOLFOX (Bokemeyer et al., 2011) or FOLFIRI (Van Cutsem et al., 2011). The improvement in overall response rate achieved by adding cetuximab to FOLFIRI was 8% and in the population treated with FOLFOX the response rate increased by 10%.

Interestingly, adding anti-EGFR agents to a regimen containing bevacizumab and a chemotherapy core did not improve the response rates toward such combination therapies (Tol et al., 2009; Hecht et al., 2009). No clinical study investigated the efficacy

of anti-EGFR agents as monotherapies in chemonaive CRC patients. Thus our results cannot be compared directly with any of the clinical studies. Moreover, clinical studies use different criteria to determine response (RECIST criteria) and the quoted response rates refer mostly to the overall response rate that includes complete response (CR: disappearance of all target lesions) and partial response (PR: at least a 30% decrease in the sum of the longest diameter of target lesions) according to the RECIST criteria. A comparison of the results obtained in chemonaive xenograft models of all UICC stages with clinical trial is, therefore, very challenging.

4.5.4. Overall Mutation Rate vs. Response to the Treatment

As discussed in chapter 4.4.1, a prevalence of oncogenic mutations in the treated subgroup of 67 models was observed. If analyzed separately, this holds true only for KRAS substitutions in codon 12, but not for other mutations in KRAS or alterations found in the BRAF or PIK3CA genes. A possible shift resulting in an increased frequency of the oncogenic mutations among the treated models would not enrich the treated subset in responders. In contrary, analyzed mutations are not only known to be oncogenic, but are associated with resistance to EGFR-targeted treatment. Therefore a higher mutation rate should result in a larger subset of resistant cases. Nevertheless the response rate of 27% obtained with cetuximab monotherapy in the panel of 67 xenograft models derived from chemonaive primary tumours of patients with all four UICC stages was higher in comparison to clinical studies in which only refractory mCRC patients or chemonaive mCRC patients were treated.

Oxaliplatin and bevacizumab, on the other hand, showed low efficacy in the treatment experiments. The response rates of 3% for bevacizumab and 6% for oxaliplatin are comparable with those obtained in the reported clinical trials, which tested the effect of single-agent therapies. Unfortunately, our understanding of the molecular background of resistance to bevacizumab and oxaliplatin is limited. In addition, there are no predictive biomarkers for the two agents. Mutations in KRAS, BRAF or PIK3CA that are linked with resistance to anti-EGFR treatment have not been connected with the response to bevacizumab or oxaliplatin.

4.5.5. Benefit from Cetuximab According to the CRC Stages

Interestingly, there was no association between the response to the anti-EGFR treatment and UICC stage of disease (p=0.1955, Chi-square, df: 4.495, 3). To the best of my knowledge there are no reports on cetuximab efficacy in patients with colorectal cancer of stage I and II. The highest response rate in our setting was achieved in stage IV (44%) and the lowest in stage III (14%) (Tab.3.9). However, only nine models derived of stage IV patients were included in the set of 67 models, out of these four xenografts showed response to cetuximab. In the clinic cetuximab is applied predominantly to patients with metastatic colorectal cancer (stage IV), but our representation of this stage is not sufficient to draw accurate conclusions.

Stage III tumours constituted over 40% of the treated xenograft models. No benefit from the addition of cetuximab in the adjuvant setting (stage III) could be seen in a large randomized phase III clinical trial (N0147) that enrolled 2664 patients. Resistance to anti-EGFR treatment in stage III patients was independent of the KRAS mutation status (Alberts et al., 2012). The N0147 trial, however, investigated a potential benefit from cetuximab added to FOLFOX4 in comparison to the FOLFOX alone and therefore required benefit from cetuximab over an established chemotherapy regimen. Cetuximab efficacy was, furthermore, tested only in the resected stage III colon cancer patients. Because of the microscopic cancer cells that remained after tumour resection, it is challenging to prove a benefit from the treatment in such patients.

4.5.6 Effect of Bevacizumab among Different CRC Stages

Not only cetuximab, but also bevacizumab failed to maintain its beneficial effect observed in patients with metastatic colorectal cancer (stage IV) when tested in the adjuvant setting in a large clinical trial. Administered to stage II and III CRC patients, bevacizumab showed no improvements in terms of response rates and overall survival in two randomized population based studies: CO-8 and AVANT (Allegra et al., 2011; de Gramont, 2011). Addition of bevacizumab was actually detrimental to the efficacy of chemotherapy alone. Similar to the N0147, trial both of the studies were designed to test postoperative efficacy of mAb in patients with stage II and III colon cancer with an aim to provide evidence for superiority of bevacizumab addition over chemotherapy regimen alone. In our xenograft study by applying a threshold of T/C<20% only two models were observed to respond to bevacizumab as monotherapy. Both responder xenografts were derived of stage III patients. If we adjust the T/C threshold retrospectively and define the responders with T/C ratio <25%, additional nine bevacizumab responders will be recognized. Five of them represent stage III tumours. Applying the same retrospective adjustment of the T/C ratio threshold in case of cetuximab would clearly result in an

increased response rate. Biomarkers established in the clinic would, however, no longer apply in our setting of xenograft models. Such shift of the T/C ratio threshold for cetuximab is therefore unjustified. Lack of biomarkers associated with the response to bevacizumab restrains the retrospective optimization of the chosen threshold for the anti-VEGF treatment.

4.5.7 Structural differences of the Four CRC Stages - Impact on the Response to the Treatment

As already discussed, an addition of cetuximab or bevacizumab to the FOLFOX scheme in patients with stage II or stage III colorectal cancer did not result in any increase of progression free survival or overall survival in large clinical trials. Why a simple translation of efficacy of these two targeted antibodies from advanced metastatic colorectal cancer into the adjuvant setting did not work? The most probable explanation for this failure is a difference in the structure of the tumour in stage II/III versus stage IV (Sobrero and Di Benedetto, 2012). In large T4 tumours of stage II, adjuvant treatment is applied to remove residual tumour mass after the surgery. In stage III additional chemotherapy is used to inhibit the migration of potential microscopic tumour cells and the spread of tumour cells from potential positive lymph nodes, which were not removed during surgery. In stage IV colorectal cancer the main goal of the treatment is the removal of the primary tumour mass by surgery, and if possible, the resection of single isolated metastases. In the majority of cases, however, metastases are too large or too numerous and the palliative therapy is administered to shrink the metastases mass to such extend that resection of the remaining metastases will be possible. Even assuming identical genetic and epigenetic background among different CRC stages, the adjuvant setting (stage II and III) and stage IV colorectal cancer seem to be mechanistically different diseases with a difference in sensitivity of macromestases and micromestases as exposed to the treatment. Therefore an assumption that stage II/III and metastatic stage IV disease should be treated using similar targeted agents seems incorrect.

Such considerations indicate a possible limitation of the xenograft models in drug efficacy testing. The set of 133 xenografts was derived from primary tumours only. This concept is supported by the fact that primary tumour specimen is the only material routinely available in the clinical practice. Recently another panel of CRC patients-derived xenografts was created (Bertotti et al., 2011). Engrafted tissues were however derived, not from primary tumours, but from CRC liver metastases. Forty four of these metastatic models were treated with cetuximab resulting in tumour shrinkage in 5 cases (11%), disease stabilization in 14 cases, and disease progression in the remaining 28 cases. In comparison, a response rate of 27% was achieved in the primary tumours in our panel of

models. In the study of Bertotti et al. cetuximab response was defined as regression of at least 50% in the tumour volume compared to the baseline tumour volume. Using this criterion the response rates were inferior in comparison to the results of our study in which more stringent criteria were applied. Moreover, there were no clinical data regarding potential previous treatment of the patients (e.g. adjuvant chemotherapy) from whom the liver metastases were resected. The differences in the design of the study, as well as the limited number of stage IV CRC tumours treated in our setting, make a comparison between the two studies difficult. To investigate whether the actual origin of the tumour tissue (primary/metastasis) makes a difference in terms of response to the treatment, an approach of comparing the response rate in both tissues originating from the same patients would be of high interest. Nevertheless, due to the lack of primary tumour tissue and matched liver and lung metastases, such treatment experiment could not be performed to date. It is, however, possible that the difference in the efficacy of cetuximab between the two xenograft panels is caused not by the engrafted material, but by the dose and the treatment schedule. In the study performed by Bertotti and colleagues cetuximab was administered twice a week at a dose of 20 mg/kg, while in our setting daily doses of 50 mg/kg were given.

4.5.8 Response and the Used Doses

Therapeutic protocols in humans avoid nearly toxic maximum tolerated doses (MTD). For the monoclonal antibodies cost-effective doses were established in our study. The use of the relatively high dose of cetuximab may be therefore a reason for the high response to cetuximab in comparison to the clinic and the study of metastatic xenograft models. It could lead to potentially false positive tumour responses in mice and to over-interpretation of the preclinical activity of cetuximab. That does not hold for the other two tested compounds oxaliplatin and bevacizumab. Response to both bevacizumab and oxaliplatin was observed in few models only. In xenograft models for majority of the agents a significantly higher MTD can be established than in human patients leading potentially to larger therapeutic effects in xenografts than in patients. In some cases, however, a lower or similar MTD applied to both patients and the models (Kerbel, 2003). Due to the different mechanisms of actions of the tested drugs and variety of factors that influence their efficacy, a comparison between the three tested agents is impossible. There is, therefore, no clear evidence that the doses applied in the treatment experiment improved the response rates in our study.

4.6 BIOMARKERS OF RESPONSE TO ANTI-EGFR mAb IN THE SET OF 67 TREATED XENOGRAFT MODELS

4.6.1 Mutation Status and Response to the anti-EGFR Treatment

Possibility of the false positive tumour responses seems to be unlikely as well in the context of predictive biomarkers analyzed in the set of 67 treated xenografts. For the two tested agents: bevacizumab and oxaliplatin no such approved biomarkers exist, moreover underrepresentation in the group of responding models prevented discovery of new ones. Due to pragmatic reasons the biomarker evaluation effort of this study was focused on cetuximab. KRAS and BRAF mutation were already known markers for resistance to cetuximab. Nevertheless, until now mutations in codon 12 and 13 were occasionally reported as well within responders to anti-EGFR treatment (Peeters et al., 2010; Sartore-Bianchi et al., 2009b; Benvenuti et al., 2007; Karapetis et al., 2008a). In our panel of 67 treated xenografts, three out of the total of 18 cetuximab responding xenograft models (17%, 95% CI: 4-41%) were found to carry KRAS mutations (Tab.3.10). Two of them harboured a substitution in codon 13 (G13D; 38G>A), which according to the COSMIC database, accounts for app. 20% of all mutations found in the KRAS gene. Our observation is in agreement with the clinical data published by De Roock et al. indicating that codon 13 mutants may actually benefit from anti-EGFR treatment (De Roock et al., 2010b). Functional differences between the mutations in codon 12 and 13 of the KRAS gene were previously investigated using in vitro approach (Guerrero et al., 2000). Specimens carrying the mutation in codon 12 formed colonies of increased cell density, induced spontaneous anchorage-independent growth, and demonstrated reduced predisposition to enter apoptosis in comparison to the KRAS codon 13 mutants (Guerrero et al., 2000). Such differences in the oncogenic effect of KRAS mutations in respect to their position support an observation that codon 13 mutants are more sensitive to anti-EGFR treatment than KRAS codon 12 mutants. Despite the conflicting data for codon 13, KRAS mutations are the only approved biomarkers for predicting response to the anti-EGFR treatment to date.

Nevertheless, most of the clinical data also demonstrate that BRAF mutation V600E is associated with resistance to cetuximab treatment. Results of numerous studies as well as our findings provide proof that patients harbouring BRAF V600E mutation derive no clinical benefit from anti-EGFR targeted agents (Di Nicolantonio et al., 2008; Loupakis et al., 2009; De Roock et al., 2010a; De Roock et al., 2011).

While in case of BRAF, the results are unambiguous, the role of PIK3CA mutations as markers of resistance to cetuximab is still controversial. Results of the study conducted in 2009 by Prenen and colleagues neglect the correlation between PIK3CA mutation

status and the benefit from cetuximab treatment. On the other hand, results of a population based study conducted in 773 patients indicate that only PIK3CA exon 20 mutations and not mutations in exon 9, are significantly associated with resistance to anti-EGFR therapy (De Roock et al., 2010a). In this setting only one model carried an exclusive PIK3CA mutation in exon 9 (E545K) and was classified as resistant to cetuximab with a T/C (treated to control) ratio of 34%. Additional six PIK3CA mutations (four in exon 9 and 2 in exon 20) were found in xenograft models that already carried KRAS mutations. The second mutation in the PIK3CA gene seems to enhance an oncogenic effect of the KRAS mutation that results in the activation of the signaling pathway regardless of EGFR blocking. The mean of T/C ratios in the group of non-responders carrying two mutations, one in KRAS and the second in PIK3CA, was 77% (95% CI: 57-89%) in comparison to 57% (95% CI: 48-64%) in the non-responders with KRAS mutation only. The T/C ratios in the group of non-responders carrying two mutations appear higher than those of non-responders carrying one mutation. The difference, however, does not reach statistical significance as the 95% confidence intervals overlap.

Despite the predictive value of the analyzed mutations, there were 11/49 (22% CI: 11-37%) non-responders that did not harbour a mutation in any of the three genes KRAS, BRAF and PIK3CA. Resistance in some of these cases could be explained by the presence of other activating mutations that were not investigated in this study. To the current knowledge no other common mutations are related with the response to anti-EGFR mAbs. Other mutational hotspots in the APC, CTNNB1, and TP53 genes, which are frequently mutated among CRC patients, were also assessed. Our data suggested that APC, CTNNB1 and TP53 mutations in the analyzed hotspots are not associated with responsiveness to cetuximab or were not frequent enough to prove their predictive value in the group of 67 xenograft models. Although mutations in NRAS were reported to have a similar impact on the efficacy of the anti-EGFR mAb that KRAS mutations, NRAS mutations occur relatively rare (2 - 4%) (De Roock et al., 2010a; Maughan et al., 2011). Thus NRAS mutations cannot explain every resistant case.

A classificator composed, additionally to KRAS, BRAF and PIK3CA, of a number of rarely mutated genes would require disproportionally more effort in terms of analyses, by a chosen method, in comparison to its contribution in the stratification of the patients. Moreover, due to the small subset of positive cases, proving efficacy of such rare biomarkers requires a great number of samples to reach statistical significance.

4.6.2 RNA Expression and Response to Cetuximab

We decided to search for potential new biomarkers also on the RNA level using on the U133 Plus 2.0 Affymetrix microarrays, which allowed a genome-wide transcript search. Discovery effort for classifying RNA expression signatures was performed using a nested bootstrap approach. The best RNA expression signature found in xenografts has a sensitivity of nearly 90% and a specificity of 85%. A signature obtained by GEP on the Affymetrix U133 Plus 2.0 microarrays in the xenograft models confirmed the importance of EGFR ligands - AREG and EREG expression in response to cetuximab, observed previously in the real-time PCR analyses (AREG: PCC= 0.65; EREG: PCC= 0.63). An inverse experiment performed with a purpose of translating results obtained on the array into real-time PCR approach failed. Correlation between response and RNA expression of LOC158960 (the first among the best classifying genes) achieved a PCC of – 10% and of PTPRF (place 6 among the best classifying genes) a PCC of 24% using real-time PCR methods. Thus the Affymetrix array results could not be validated by real-time PCR. A possible reason is the difference in the binding sites and the number of probes used on Affymetrix GeneChip and allele-specific probes for the genes of interest. The overall low expression levels of LOC158960 measured by allele-specific probes in the real-time PCR approach could also negatively affect the results and influence the difference in expression levels by the process of normalization.

4.6.3 microRNA Expression and Response to Cetuximab

FFPE is a standard method used in routine pathological practice to preserve tumour specimens. It leads, however, to a degradation of nucleic acids. Therefore, microRNAs that possess a high degree of stability are an attractive target in the molecular analyses of tumour FFPE tissue samples and their potential for predicting response to cetuximab was also investigated. This class of short ~22 nucleotide ribonucleic acid molecules plays a role in the post-transcriptional regulators that bind to multiple target-mRNAs. Binding to the complementary sequences in the 3’ UTR usually results in translational repression, target degradation, and in gene silencing. microRNAs are predicted to regulate app. 60% of all human genes. Since their discovery in 1993 more than 1.700 unique mature human micro-RNAs have so far been identified (miRBase v18). In 2011 521 new microRNA sequences were identified, resulting in the increase in the number of known unique mature human miRNA by 43% from 1212 to 1733. This number is growing constantly.

Bioinformatic analysis showed that the best microRNA signature included all 1404 informative probesets and reached sensitivity of 82.3% and a specificity of 73.1%. In general, shorter microRNA signatures e.g. with 54 probesets had only a limited predictive capacity reaching a sensitivity of 63.4% and a specificity of 64.4%. In comparison, a

short RNA marker containing signature of 54 probesets generated on the Affymetrix U133 Plus 2.0 array had a sensitivity of 85.2% and a specificity of 81.1%.

It is unlikely that microRNAs are less suitable for response prediction than mRNAs. However, it seems that the total number of markers after normalization is critical for predicting response to drugs, particularly if absolute differences in expression of microRNA or mRNA markers are relatively small between the responder and non-responder groups. The GeneChip Human Genome U133 Plus 2.0 Array contains 54.000 probesets representing 47.000 transcripts and variants of only human genes, which is app. 20 times the number of probesets contained on the microRNA array.

Interestingly, taking under account our still limited knowledge and understanding of the function of particular microRNAs, surprisingly large fraction of the best performing microRNAs was already linked with regulation of either EGFR pathway (miR-125, let-7 family, miR-99b, miR-143, miR-145) or with other events known to contribute to colorectal tumorigenesis like loss of PTEN (miR-195) or TGF-β1 signaling (miR-224) (Monzo et al., 2008; Ng et al., 2009; Slaby et al., 2009; Volinia et al., 2006). This functional link seems to confirm the potential of microRNA signatures. The greatest advantage of the microRNA in comparison to mRNAs is their robustness in degraded RNA samples. Therefore microRNAs are of great interest in clinical application that uses standard FFPE tissue preservation.

4.6.4 Combining the Mutation Status with RNA Expression Levels has the Highest Accuracy for Predicting Response to Cetuximab

Neither the mRNA nor the miRNA signature obtained with Affymetrix array technology achieved high accuracy. In addition, the efficacy of the mRNA signature could not be confirmed in the set of corresponding primary tumours. Among the best classifying genes from this mRNA signature were, however, AREG and EREG, previously reported to be linked with the response to the anti-EGFR treatment.

Real-time PCR measurements confirmed a significant association of high expression of the two EGFR ligands and sensitivity to cetuximab in the set of xenografts (AREG: PCC=0.72; EREG: PCC=0.75). Measurement of the AREG and EREG RNA expression in the corresponding snap-frozen and FFPE tissues of original primary tumours indicates that the expression level of analyzed genes is to some extent preserved (snap frozen: AREG PCC: 0.60; EREG PCC: 0.75; FFPE: AREG PCC: 0.55, EREG PCC: 0.57). Moreover, the higher predictive power of AREG and EREG mRNA expression level was observed in the models pre-selected by mutation analysis.

To avoid further technical challenges a sequential analysis of mutation and mRNA expression by real-time PCR was applied. Such combination of the highest accuracy seemed also to be preserved in the primary tissue of the matched patients' tumours. A comparison of the current biomarkers and their possible combinations measured by the real-time PCR is shown in Tab. 4.1.

Tab.4.1. *Cross tabulation table shows the relation between response towards cetuximab and - Predictor 1: mutation status in codon 12 and 13 of KRAS (standard patient selection procedure); Predictor 2: all mutation in KRAS, BRAF and PIK3CA; Predictor 3: all mutations in KRAS (excluding codon 13 mutations), BRAF and PIK3CA; Predictor 4: all mutations in KRAS (excluding codon 13 mutations), BRAF and PIK3CA, combined with RNA expression of AREG and EREG.*

		Observed Response		
	Predicted Response	Yes	No	Sums:
Predictor 1	WT	15	23	38
	Mut	3	26	29
	Sums:	18	49	67
Sensitivity/Specificity:		83% (61%-94%)	53% (39%-66%)	
Predictor 2	WT	15	11	26
	Mut	3	38	41
	Sum	18	49	67
Sensitivity/Specificity:		83% (61%-94%)	78% (64%-87%)	
Predictor 3	WT+ c.13	17	15	32
	Mut	1	34	35
	Sum	18	49	67
Sensitivity/Specificity:		94% (74%-99%)	69% (55%-80%)	
Predictor 4	WT+ c.13	17	7	24
	Mut + AREG,EREG	1	42	43
	Sum	18	49	67
Sensitivity/Specificity:		94% (74%-99%)	86% (73%-93%)	

Standard testing is currenty routinely performed only for KRAS mutations in codon 12 and 13. No mutations in these two KRAS codons were found in the 38 out of the 67 treated xenograft models. According to the standard approach, anti-EGFR mAb would be administered to all of them, while only 15 out of them would respond to cetuximab and 23 would not. Such patients' stratification results in a positive predictive value (PPV) of only 0.39 and contributes to the unnecessary toxicity and costs. A negative predictive value (NPV) of 26/29 = 0.90 indicates that only three out of potential responders would not receive the treatment according to the current guidelines (Tab.4.1, predictor 1).

If we include in the classifier all the mutations in KRAS, BRAF and PIK3CA, the PPV increases to 15/26 = 0.58 (Tab.4.1, predictor 2). A predictive panel composed of all

analyzed mutations would recognize additional 12 non-responders and prevent over-treatment in these patients. Seven of these 12 non-responders carry BRAF V600E mutations indicating that this mutation should be now considered, alongside with KRAS mutations, as a criterion to exclude the patient from the treatment with anti-EGFR mAbs. Four additional KRAS mutations - two in codon 61 and two in codon 146 were found in our setting. All of these mutants did not benefit from the treatment with cetuximab. Only one model carries a single PIK3CA mutation. Although no response was observed in this model, a larger study is needed to decide whether PIK3CA mutations are strongly associated with the lack of benefit under anti-EGFR treatment, as the results of clinical studies are conflicting. The NPV of the second predictor is 38/41 = 0.93.

An assumption that KRAS codon 13 mutants benefit from the treatment would lead to the further refinement of the classifier and an increase in the NPV to 34/35 = 0.97 (Tab.4.1, predictor 3). That means that only one potential responder would not obtain the treatment. On the other hand, as four codon 13 mutants showed no response in our setting, under such assumption, the PPV drops to 17/32 (0.53).

A combination of the latter classifier with determination of RNA expression of AREG and EREG results in the best potential predictive classifier with a sensitivity of 94% and a specificity of 86%. The PPV of this predictor is 17/24=0.71 as the expression level of the two EGFR ligands indicates which of the KRAS codon 13 mutants are unlikely to respond to cetuximab (Tab.4.1, predictor 4). The resulting NPV is 42/43 =0.98 meaning that only one patient recognized by the predictive panel as resistant would be an actual responder (Fig.4.1). To my best knowledge, no guideline concerning the AREG and EREG expression cut-off exists so far. As measured by real-time PCR, expression data needs normalization and as well here no standard procedures were yet established. Such technical difficulties lead to the challenges in incorporating of the AREG and EREG in the standard testing procedure prior to the therapy, although their clear predictive value was observed by us and by others (Baker et al., 2011; Jacobs et al., 2009; Khambata-Ford et al., 2007).

The retrospective character of this study is manifested i.a. by the optimized selection of the cut-off in the expression analysis of AREG and EREG. Such approach may result in a higher accuracy of the predictive panel. Although a potential shift in the prevalence of the mutated tumours in the subset of 67 treated models seems to have no effect on the cetuximab response rate, it may also affect the predictive value of established classifier. As analyzed oncogenic mutations are linked with the resistance to anti-EGFR, their high frequency could enrich the fraction of recognized non-responders resulting in an improved specificity.

In spite of the fact that the presented values are found in a retrospective analysis, an agreement of our data with the previous findings strongly demonstrates their clinical potential. I therefore think it is prudent to adopt a sequential patients' stratification approach in which mutations in KRAS codons 12, 61 and 146 would be analyzed as first. In the group of patients wild-type in these KRAS hotspots, BRAF mutation analysis should result in the further identification of the resistant cases. Underrepresentation of single PIK3CA mutations in our panel does not allow conclusions about the usefulness of PIK3CA in improving sensitivity of the classifier. Although, not analyzed in our setting, strong evidence supports consideration of NRAS mutations in the standard protocol to select patients prior to the admission of the anti-EGFR mAb. Finally, an effort should be made to adjust and include the analysis of AREG and EREG expression and enable incorporation of gene expression biomarkers in the last step of the selection procedure. Such analysis, performed in the patients that are wild-type in the BRAF, NRAS and codon 12, 61 and 146 of the KRAS gene should finally enrich the fraction of the recognized non-responders. In conclusion, adjusting for their retrospective character, our analyses deliver an informative value of the priorities that should be set in the standardized selection of the CRC patients to be treated with anti-EGFR mAb.

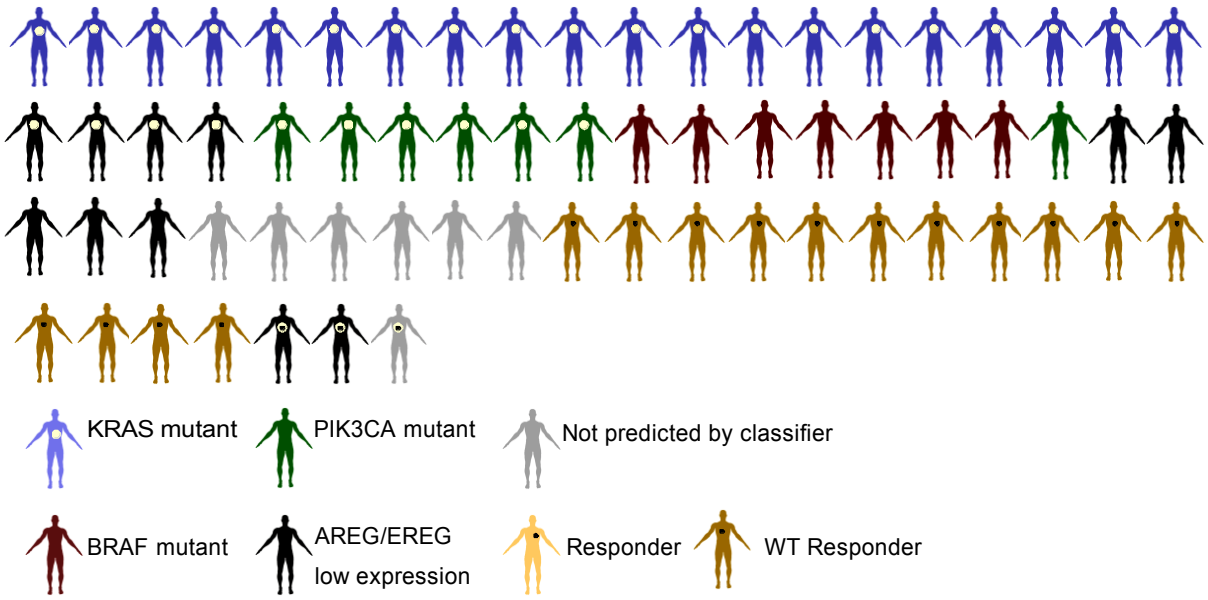


Fig.4.1. *A graphic illustration of the population corresponding to the 67 xenograft treated with cetuximab and the prediction of response by the biomarkers included in the classifier: KRAS, BRAF and PIK3CA mutations and expression level of AREG and EREG (modified:Sartore-Bianchi et al., 2009a).*

4.7 TRANSLATION OF THE RESULTS OBTAINED IN THE PRECLINICAL XENOGRAFT MODELS INTO THE CLINIC

A five year follow-up of the patients from the MSKK study, from whom the xenografts were derived, does not yet exist. Thus a direct comparison of the drug efficacy observed in preclinical xenograft models with an actual response of the patients in clinical routine is, to date, unfortunately not possible. Consistency between the predictive value of the biomarkers established in the clinical trials and the response rates in the panel of xenografts has been, however, demonstrated. The panel of 133 patient-derived xenografts seems, therefore, to be an attractive model not only as a novel approach for the discovery of predictive biomarkers, but also for advanced testing of novel anti-cancer agents.

Although patients-derived xenografts are not ideal models, they have apparent advances over the other methods that are in a wide use in the preclinical approach such as in vitro and in vivo studies using the cancer cell lines, or transgenic mice (Sausville, E.A. et al.; 2006). Patient-derived xenografts represent actual, individual, tumours and, although they derive from a small part of the neoplastic tissue, these xenografts seem to highly resemble the morphologic and molecular characteristics of original primary tumours. On the other hand, there are certain limitations concerning the structure of engrafted tumours. Substitution of the human stroma by the murine cells and the revascularization by penetration of implants with proliferating host blood vessels distinguish engrafted tissues from their corresponding primaries. Taking into account the differences between human tumours and xenograft models derived from transplantation of human tumour tissue, an optimal experimental design needs to be developed to unravel the full potential of novel drugs in treatment experiments in a multitude of xenograft models that allow mimicking phase II clinical trials.

A proper treatment plan and dosing scheme are crucial to obtain a sufficient exposure to the experimental drug or drug combination and its balanced distribution. Near-toxic dosing is undesirable, thus administration of maximum doses to the xenograft models may result in the overestimation of the efficacy of tested compounds in humans and lead to unnecessary costs. Other important component of the experimental design is a number of xenograft models to be used in treatment experiments to reach statistical significance and the sufficient number of negative controls. One also needs to be careful with determining the proper endpoints of the study as the weight or volume measurements, particularly of the small tumours are highly susceptible to the operators' error (Hollingshead, 2008).

Assuming an optimal experimental design under all of mentioned considerations, the panel of xenografts is suitable to examine pharmacokinetics, metabolism and mechanism of action of the novel therapeutic compounds. The advantage of the approach is that one can test a novel targeted compound in any combination with other targeted agents without having to prove efficacy of this combination in human clinical trials. The same applies to the combination of targeted agents with chemotherapeutics. A minor shift in favor of tumours that carry oncogenic mutations, particularly in KRAS codon 12, was observed in the 67 models used in the treatment experiment and indicates that a careful selection of models should be applied in the future experiments. The panel of 133 xenograft models can be use in drug development and the development of companion diagnostics as they mimic the individual tumours as well as a random subset of the CRC patients' population.

Patients' selection recently entered the clinical practice with an establishment of such predictive biomarkers as KRAS in CRC, HER-2 expression prior to the treatment with trastuzumab in breast cancer and EGFR mutation analysis to predict response to EGFR tyrosine kinase inhibitors (erlotinib, gefitinib) in non-small cell lung cancer (NSCLC). Discovery of predictive biomarkers, so far introduced late in the drug development process, is being substituted by the efficacy studies that are in fact driven by the biomarker hypothesis. Determination of various genetic and epigenetic alterations such as mutations, SNPs, copy-number alterations, altered gene expression or methylation patterns etc., that may affect the efficacy of a drug is still needed for our better understanding of the mechanism of action of tested compounds. Further molecular characterization of the panel of 133 xenograft models is required and is already being executed. The panel of 133 CRC patients-derived xenografts is, up to our best knowledge, the largest available collection of the primary, chemonaive CRC models. It represents patients' population, therefore reflects a whole variety of genotypes making them suitable for testing of wide spectrum of anti-cancer agents. Patients-derived xenografts are an answer to the changing strategy from a 'one-size fits all' approach to the requirements of personalized medicine.

Not long ago, a concept of engrafting tumour tissue of a patient, testing tumour response to the various therapies on the established xenograft model and using the results as an actual deciding factor in the choice of the most beneficial therapy for the particular patient, seemed to be utopian. Nevertheless, a recent pilot study tested 63 drugs in 232 treatment regimens in 14 patient-derived xenograft tumours of various indications (Hidalgo et al., 2011). For 12 out of 14 patients an effective treatment regimen was identified on the basis of the observation of the activity of different drugs in the

personalized xenograft generated from the patient's resected tumour tissue. The treatments selected for each patient were not obvious and would not have been the first choice. According to the efficacy data obtained in the xenograft models, patients received prospectively guided treatments. An objective response rate of 88% was reported with 11/14 patients achieving a partial response. Despite such promising results, a broad clinical application of such approach needs, however, to overcome several hurdles. High cost of such experiments is a potential limiting factor. Moreover, not all tumour tissues can be successfully engrafted and even in the best conditions app. 25-30% of implants fail. Limitations such as time required for establishing and conducting treatment experiments in the model are inevitable, given that particularly advanced cancer stages require immediate decisions about the treatment regimen.

A large panel of 133 xenografts reflects a wide spectrum of tumours. A molecular characterization of the panel was already performed in terms of mutation status, mRNA and miRNA expression patterns. Further molecular analyses on both: genetic and epigenetic levels are under progress in order to provide a detailed characterization of the xenograft models. Such deep molecular characterization would allow finding a model matching the properties of the particular patients' tumour. Results of the treatment experiments with various therapeutic regimens performed in the chosen model could influence decision making for the patient and, if successful, improve the response rate and outcome of the cancer patients. The panel of patient-derived CRC xenograft models is, therefore, a potential useful tool that contributes not only to the discovery of novel compounds and identification of predictive biomarkers. It has also a potential to influence the treatment decisions and so to enable a step toward personalized medicine.

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Declaration

I hereby declare that, except where otherwise stated, this dissertation entitled: "A novel approach to develop predictive biomarkers. Prediction of response to anti-EGFR therapy in a large panel of patient-derived colorectal cancer xenograft models." is the result of my own work. No part of this dissertation was submitted at any University for a diploma, degree, or other qualification.

Paulina Pechańska, Berlin, September 2013

Appendix

Tab.S1. *International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) Version for 2010; colorectal cancer classification according to the localization of primary tumour*

ICD-10 class	Localization of primary tumour
C18.0	Caecum; ileocaecal valve
C18.1	Appendix
C18.2	Ascending colon
C18.3	Hepatic flexure
C18.4	Transverse colon
C18.5	Splenic flexure
C18.6	Descending colon
C18.7	Sigmoid colon, excl.: rectosigmoid junction
C18.8	Overlapping lesion of colon
C18.9	Colon, unspecified
C19	Rectosigmoid junction
C20	Rectum incl.: rectal ampulla
C21.0	Anus, unspecified
C21.1	Anal canal, *a*nal sphincter
C21.2	Cloacogenic zone
C21.8	Anorectum, anorectal junction

Tab.S2. *Cancer Staging according to the Seventh Edition of Cancer Staging Manual by American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). T0 – no evidence of primary tumour; Tis - carcinoma in situ; T1 - tumour invades submucosa; T2 - tumour invades muscularis propia; T3 - Tumour invades through the muscularis propria into pericolorectal tissues; T4a - tumour penetrates to the surface of the visceral peritoneum; T4b - tumour directly invades or is adherent to other organs or structures; N0 - no regional lymph node metastasis; N1 - metastasis in 1–3 regional lymph nodes; N1c - tumour deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis; N2 - metastasis in 4 or more regional lymph nodes; N2a - metastasis in 4–6 regional lymph nodes; N2b - metastasis in 7 or more regional lymph nodes; M0 - No distant metastasis; M1a - metastasis confined to one organ or site; M1b - metastases in more than one organ/site or the peritoneum (http://www.cancerstaging.org)*

Stage	T	N	M
0	Tis	N0	M0
I	T1-2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-2	N1/1c	M0
	T1	N2a	M0
	T3-4a	N1/1c	M0
IIIB	T2-3	N2a	M0
	T1-2	N2b	M0
	T4a	N2a	M0
IIIC	T3-4a	N2b	M0
	T4b	N1-2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

Tab.S3. *Sequences of the primers and probes of the allele-specific assays (Applied Biosystems). Reporters 1 are labeled with VIC and Reporters 2 are labeled with FAM. All probes use Black Hole Quencher (*due to the courtesy of Prof. S. Tejpar)*

Assay ID	Target Mutation nt	Target Mutation aa	Forward Primer Seq.	Reverse Primer Seq.	Reporter 1 Sequence	Reporter 2 Sequence
KRAS_ex2-121a	34 G>A	G12S (Gly - Ser)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCTT	TTGGAGCTGGTGGCGTA	TAGTTGGAGCTAGTGGCGTA
KRAS_ex2-121t	34 G>T	G12C (Gly - Cys)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCTT	TTGGAGCTGGTGGCGTA	TTGGAGCTTGTGGCGTA
KRAS_ex2-121c	34 G>C	G12R (Gly - Arg)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCTT	TTGGAGCTGGTGGCGTA	TTGGAGCTCGTGGCGTA
KRAS_ex2-122a	35 G>A	G12D (Gly - Asp)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCTT	TTGGAGCTGGTGGCGTA	TTGGAGCTGATGGCGTA
KRAS_ex2-122t	35 G>T	G12V (Gly - Val)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCTT	TTGGAGCTGGTGGCGTA	TTGGAGCTGTTGGCGTA
KRAS_ex2-122c	35 G>C	G12A (Gly - Ala)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCTT	TTGGAGCTGGTGGCGTA	TTGGAGCTGCTGGCGTA
KRAS_ex2-131t	37 G>T	G13C (Gly - Cys)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCTT	TTGGAGCTGGTGGCGTA	TTGGAGCTGGTTGCGTA
KRAS_132a*	38 G>A	G13D (Gly - Asp)	AGGCCTGCTGAAAATGACTGAATAT	GCTGTATCGTCAAGGCACTCT	TGGTGGCGTAGGCA	CTGGTGACGTAGGCA
BRAF	1799 T>A	V600E (Val - Glu)	CTACTGTTTTCCTTTACTTACTACACCTCAGA	ATCCAGACAACTGTTCAAACTGATG	CTAGCTACAGTGAAATC	TAGCTACAGAGAAATC
KRAS_146a	436 G>A	A146T (Ala - Thr)	GGCTCAGGACTTAGCAAGAAGTTAT	GCAGAAAACAGATCTGTATTTATTTCAGTGT	TCTTGTCTTTGCTGATGTT	TCTTGTCTTTGTTGATGTT
PIKex9-1624	1624 G>A	E542K (Glu - Lys)	AGCTCAAAGCAATTTCTACACGAGAT	GCACTTACCTGTGACTCCATAGAAA	CCTCTCTCTGAAATCA	CCTCTCTCTAAAATCA
PIKex9-1633	1633 G>A	E545K (Glu - Lys)	TCAAAGCAATTTCTACACGAGATCCT	GCACTTACCTGTGACTCCATAGAAA	CTCTCTGAAATCACTGAGCAG	CTCTGAAATCACTAAGCAG
PIKex20-3140	3140 A>G	H1047R (His - Arg)	GCAAGAGGCTTTGGAGTATTTCATG	GCTGTTTAATTGTGTGGAAGATCCAA	CCACCATGATGTGCATC	CACCATGACGTGCATC
CTNNex3-134	134 C>T	S45F (Ser -> Phe)	GGAATCCATTCTGGTGCCACTAC	CCTCTTCCTCAGGATTGCCTTT	CCACTCAGAGAAGGAG	CACTCAGAAAAGGAG
CTNNex3-121	121 A>G	T41A (Thr -> Ala)	CTGGCAGCAACAGTCTTACCT	GGATTGCCTTTACCACTCAGAGAAG	CTGGTGCCACTACCACA	TGGTGCCACTGCCACA
TP53ex5-524	524 G>A	R175H (Arg ->His)	ACAGCACATGACGGAGGTT	CTGCTCACCATCGCTATCTGA	TGAGGCGCTGCCC	TGAGGCACTGCCC

TP53ex7-742	742 C>T	R248W (Arg -> Trp)	AACTACATGTGTAACAGTTCCTGCAT	GAGTCTTCCAGTGTGATGATGGT	TGGGCCTCCGGTTCA	TGGGCCTCCAGTTCA
TP53ex7-743	743 G>A	R248Q (Arg -> Gln)	AACTACATGTGTAACAGTTCCTGCAT	CCAGTGTGATGATGGTGAGGAT	CATGAACCGGAGGCC	CATGAACCAGAGGCC
TP53ex8-818	818 G>A	R273H (Arg -> His)	CTTGCTTCTCTTTTCCTATCCTGAGT	CTGTGCGCCGGTCTCT	TGAGGTGCGTGTTTGT	TTGAGGTGCATGTTTGT
TP53ex8-844	844 C>T	R282W (Arg -> Trp)	GCTTTGAGGTGCGTGTTTGTG	GGTGAGGCTCCCCTTTCTTG	TGCGCCGGTCTCT	TGCGCCAGTCTCT
APCex16-4348	4348 C>T	R1450* (Arg -> *)	CTCCACCACCTCCTCAAACAG	ACTCTCTCTCTTTTCAGCAGTAGGT	TAGGTACTTCTCGCTTGGTT	AGGTACTTCTCACTTGGTT

Tab.S4. *TaqMan Gene Expression Assays used in the expression analysis of selected target genes: AREG, EREG, PTEN, DUSP6, SLC26A3, LOC158960, PTPRF and housekeeping genes: GAPDH, RPLP0 and UBC*

Gene Symbol	Assay ID	Context Sequence	Gene Name
AREG	Hs00950669_m1	AACATGCAAATGTCAGCAAGAATAT	amphiregulin
DUSP6	Hs00737962_m1	CTACCTGGAAGATGAAGCCCGGGGC	dual specificity phosphatase 6
EREG	Hs00914313_m1	AGTCAAAACTACTGCAGGTGTGAAG	epiregulin
GAPDH	Hs00266705_g1	GGTCGGAGTCAACGGATTTGGTCGT	glyceraldehyde-3-phosphate dehydrogenase
LOC158960	Hs00863860_m1	ACCCTCCCTGATACAGGAGGAGAAA	hypothetical protein BC009467
PTEN	Hs02621230_s1	ATGCTGCACAGAAATTTTCAATTTG	phosphatase and tensin homolog
PTPRF	Hs00160858_m1	AGCTGCGTTCAGGTGCCTTGCAGAT	protein tyrosine phosphatase, receptor type, F
RPLP0	Hs00420895_gH	ACCCAGCTCTGGAGAAACTGCTGCC	ribosomal protein, large, P0
SLC26A3	Hs00995363_m1	TGTATATCGTTGGAACTGATGATGA	solute carrier family 26, member 3
UBC	Hs00824723_m1	TGATCGTCACTTGACAATGCAGATC	ubiquitin C

Tab.S5. *Basic clinical (age, gender, tumour localization, stage) and pathological (tumour classification) features of the 864 patients enrolled into MSKK and RVS studies and analyzed for mutations in KRAS, BRAF and PIK3CA. Mut No - number of detected mutations; Seq. - result of the dideoxy - sequencing of codon 12 and 13 of the KRAS gene; ICD-O - colorectal cancer classification according to the localization of primary tumour (see Tab. S2), UICC - cancer staging according to the International Union Against Cancer (see Tab. S1); F- female; M- male; WT – wild-type; Mixed Diff.with Mucin – mixed differentiation with mucin; Rest after neoadj.th. – Rest after neoadjuvant therapy*

No	patient-ID	model - ID	Tissue	Mut No	BRAF	KRAS	PIK3CA	Seq.	Study	Age	Sex	ICD-O	UICC	Classification
1	P5	-	FFPE	0	WT	WT	WT	WT	MSKK	71	F	C18.2	III	Adenocarcinoma
2	P6	-	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	47	F	C18.2	I	Adenocarcinoma
3	P10	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	58	M	C20.-	II	Adenocarcinoma
4	P11	-	FFPE	0	WT	WT	WT	-	MSKK	77	F	C18.5	II	Adenocarcinoma
5	P12	-	FFPE	0	WT	WT	WT	NA	MSKK	73	F	C20.-	I	Adenocarcinoma
6	P13	-	FFPE	1	WT	WT	1624 G>A	-	MSKK	71	F	C18.2	III	Adenocarcinoma
7	P14	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C20.-	III	Adenocarcinoma
8	P15	-	Snap-frozen	1	WT	34 G>T	WT	34 G>T	MSKK	71	M	C18.0	II	Adenocarcinoma
9	P16	-	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	72	F	C18.7	III	Adenocarcinoma
10	P17	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	75	M	C19.9	II	Adenocarcinoma
11	P18	-	FFPE	1	WT	436 G>A	WT	WT	MSKK	77	F	C19.9	II	Adenocarcinoma
12	P20	-	FFPE	1	WT	38 G>A	WT	-	MSKK	85	F	C20.-	I	Tumour in Adenoma
13	P21	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	62	F	C18.2	III	Mucinous Carcinoma
14	P22	-	FFPE	0	WT	WT	WT	-	MSKK	46	F	C18.4	II	Adenocarcinoma
15	P23	-	FFPE	0	WT	WT	WT	-	MSKK	72	F	C18.2	II	Adenocarcinoma
16	P37	-	Snap-frozen	1	WT	34 G>C	WT	34 G>C	RVS	71	M	C18.7	III	Adenocarcinoma
17	P38	-	Snap-frozen	1	1799 T>A	WT	WT	-	RVS	71	M	C18.2	I	Adenocarcinoma
18	P39	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	63	M	C18.4	III	Adenocarcinoma
19	P40	-	Snap-frozen	0	WT	WT	WT	WT	RVS	72	F	C18.0	III	Adenocarcinoma
20	P41	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	47	M	C19.-	III	Adenocarcinoma
21	P42	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	70	M	C18.0	II	Adenocarcinoma
22	P44	-	Snap-frozen	0	WT	WT	WT	-	RVS	73	M	C18.7	II	Adenocarcinoma
23	P45	-	Snap-frozen	0	WT	WT	WT	WT	RVS	57	M	C18.5	I	Adenocarcinoma
24	P46	-	Snap-frozen	0	WT	WT	WT	-	RVS	65	F	C18.6	III	Adenocarcinoma
25	P47	-	Snap-frozen	0	WT	WT	WT	-	RVS	63	M	C18.3	II	Adenocarcinoma

26	P48	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	63	M	C18.7	III	Mixed Diff.with Mucin
27	P49	-	Snap-frozen	0	WT	WT	WT	-	RVS	56	M	C18.3	II	Adenocarcinoma
28	P50	-	Snap-frozen	0	WT	WT	WT	-	RVS	46	M	C18.3	II	Adenocarcinoma
29	P51	-	Snap-frozen	2	WT	38 G>A	3140 A>G	-	RVS	76	M	C18.0	III	Adenocarcinoma
30	P52	-	Snap-frozen	1	WT	436 G>A	WT	WT	RVS	60	M	C18.2	II	Mucinous Carcinoma
31	P53	-	Snap-frozen	0	WT	WT	WT	WT	RVS	58	M	C20.9	II	Adenocarcinoma
32	P28	-	FFPE	1	WT	35 G>A	WT	-	MSKK	77	F	C18.7	II	Adenocarcinoma
33	P29	-	FFPE	0	WT	WT	WT	WT	MSKK	62	M	C19.-	I	Adenocarcinoma
34	P54	-	Snap-frozen	0	WT	WT	WT	-	RVS	44	M	C18.7	III	Adenocarcinoma
35	P55	-	Snap-frozen	0	WT	WT	WT	WT	RVS	73	M	C18.7	III	Adenocarcinoma
36	P56	-	Snap-frozen	0	WT	WT	WT	WT	RVS	52	M	C18.7	I	Adenocarcinoma
37	P57	-	Snap-frozen	0	WT	WT	WT	-	RVS	72	F	C18.7	II	Adenocarcinoma
38	P59	-	Snap-frozen	0	WT	WT	WT	WT	RVS	76	F	C18.7	II	Adenocarcinoma
39	P60	-	Snap-frozen	1	WT	34 G>A	WT	WT	RVS	60	M	C18.2	III	Adenocarcinoma
40	P62	-	Snap-frozen	1	WT	38 G>A	WT	-	RVS	70	F	C18.2	II	Adenocarcinoma
41	P63	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	58	F	C19.-	IV	Adenocarcinoma
42	P65	-	Snap-frozen	0	WT	WT	WT	WT	RVS	72	M	C18.5	III	Adenocarcinoma
43	P66	-	Snap-frozen	1	WT	35 G>C	WT	35 G>C	RVS	50	F	C18.7	III	Mixed Diff.with Mucin
44	P30	-	FFPE	1	WT	35 G>T	WT	-	MSKK	77	F	C19.-	III	Adenocarcinoma
45	P26	-	FFPE	0	WT	WT	WT	WT	MSKK	63	F	C18.2	I	Adenocarcinoma
46	P27	-	FFPE	0	WT	WT	WT	WT	MSKK	70	M	C19.9	III	Adenocarcinoma
47	P1	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	83	F	C18.2	III	Mucinous Carcinoma
48	P3	-	FFPE	1	WT	35 G>T	WT	-	MSKK	70	F	C20.-	II	Mucinous Carcinoma
49	P2	-	FFPE	1	WT	436 G>A	WT	-	MSKK	85	F	C18.2	III	Mucinous Carcinoma
50	P7	-	FFPE	1	1799 T>A	WT	WT	NA	MSKK	55	F	C18.5	III	Mucinous Carcinoma
51	P9	-	FFPE	2	WT	35 G>T	3140 A>G	WT	MSKK	64	M	C20.-	II	Mucinous Carcinoma
52	P24	-	FFPE	2	WT	38 G>A	3140 A>G	38 G>A	MSKK	52	F	C18.0	I	Mixed Diff.with Mucin
53	P32	-	FFPE	0	WT	WT	WT	-	MSKK	64	M	C18.7	II	Adenocarcinoma
54	P33	-	FFPE	0	WT	WT	WT	WT	MSKK	53	M	C20.-	III	Adenocarcinoma
55	P34	-	FFPE	0	WT	WT	WT	WT	MSKK	64	F	C20.-	I	Adenocarcinoma
56	P31	-	FFPE	0	WT	WT	WT	NA	MSKK	26	M	C18.7	III	Signet-Ring-Cell Ca.
57	P68	-	Snap-frozen	1	WT	35 G>T	WT	-	RVS	64	M	C18.0	III	Adenocarcinoma
58	P67	-	FFPE	1	1799 T>A	WT	WT	NA	MSKK	85	F	C18.4	II	Mixed Diff.with Mucin
59	P35	-	FFPE	1	WT	38 G>A	WT	-	MSKK	69	F	C20.-	III	Adenocarcinoma

60	P36	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	83	F	C18.3	IV	Synchronous CRC
61	P70	-	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	62	F	C18.0	III	Signet-Ring-Cell Ca.
62	P71	-	FFPE	0	WT	WT	WT	-	MSKK	78	M	C18.2	II	Mucinous Carcinoma
63	P72	-	FFPE	1	WT	38 G>A	WT	38 G>A	MSKK	67	F	C18.0	III	Adenocarcinoma
64	P73	-	FFPE	0	WT	WT	WT	WT	MSKK	68	M	C19.-	II	Adenocarcinoma
65	P75	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	70	M	C18.6	III	Signet-Ring-Cell Ca.
66	P77	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	73	M	C19.-	II	Adenocarcinoma
67	P78	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	52	M	C18.7	II	Adenocarcinoma
68	P79	-	FFPE	0	WT	WT	WT	WT	MSKK	68	M	C20.-	II	Adenocarcinoma
69	P80	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	64	F	C18.7	III	Adenocarcinoma
70	P81	-	Snap-frozen	2	WT	35 G>T	1624 G>A	35 G>T	MSKK	72	F	C18.5	III	Mucinous Carcinoma
71	P82	-	FFPE	0	WT	WT	WT	WT	MSKK	84	F	C18.7	II	Adenocarcinoma
72	P83	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	82	M	C20.-	III	Mucinous Carcinoma
73	P84	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	53	M	C20.-	II	Adenocarcinoma
74	P85	-	FFPE	1	WT	34 G>A	WT	34 G>A	MSKK	65	M	C18.0	III	Adenocarcinoma
75	P86	-	Snap-frozen	1	1799 T>A	WT	WT	WT	MSKK	67	M	C18.5	II	Adenocarcinoma
76	P87	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	MSKK	67	M	C18.7	III	Adenocarcinoma
77	P89	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	MSKK	76	F	C20.-	II	Synchronous CRC
78	P90	-	Snap-frozen	1	1799 T>A	WT	WT	-	MSKK	75	M	C18.7	II	Synchronous CRC
79	P91	-	Snap-frozen	0	WT	WT	WT	-	MSKK	69	F	C20.-	I	Adenocarcinoma
80	P92	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	69	M	C19.-	II	Adenocarcinoma
81	P93	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	RVS	41	F	C20.91	III	Adenocarcinoma
82	P94	-	Snap-frozen	0	WT	WT	WT	-	RVS	55	F	C20.91	I	Tumour in Adenoma
83	P95	-	Snap-frozen	1	WT	35 G>C	WT	35 G>C	RVS	60	M	C20.91	III	Adenocarcinoma
84	P96	-	Snap-frozen	1	WT	WT	1624 G>A	-	RVS	51	F	C20.-	II	Adenocarcinoma
85	P97	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	66	M	C20.91	II	Adenocarcinoma
86	P98	-	Snap-frozen	1	WT	35 G>A	WT	-	RVS	82	F	C20.91	III	Adenocarcinoma
87	P99	-	Snap-frozen	1	WT	38 G>A	WT	-	RVS	77	M	C20.91	II	Mixed Diff.with Mucin
88	P100	-	Snap-frozen	1	1799 T>A	WT	WT	-	RVS	73	F	C20.-	III	Tumour in Adenoma
89	P101	-	Snap-frozen	1	1799 T>A	WT	WT	WT	RVS	51	M	C20.91	III	Adenocarcinoma
90	P102	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	67	M	C20.-	III	Adenocarcinoma
91	P103	-	Snap-frozen	0	WT	WT	WT	WT	RVS	61	M	C20.-	III	Adenocarcinoma
92	P104	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	MSKK	70	F	C20.-	III	Adenocarcinoma
93	P105	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	MSKK	66	M	C18.7	II	Adenocarcinoma

94	P106	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	94	F	C20.-	II	Adenocarcinoma
95	P107	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	71	F	C18.7	III	Adenocarcinoma
96	P108	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	66	M	C20.-	III	Adenocarcinoma
97	P109	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	50	F	C18.4	II	Synchronous CRC
98	P74	-	FFPE	1	WT	35 G>A	WT	-	MSKK	61	F	C18.0	I	Adenocarcinoma
99	P110	-	Snap-frozen	1	WT	35 G>A	WT	-	MSKK	78	F	C18.0	III	Adenocarcinoma
100	P111	-	Snap-frozen	1	WT	35 G>A	WT	-	MSKK	66	M	C19.9	III	Adenocarcinoma
101	P112	-	FFPE	1	WT	WT	1633 G>A	-	MSKK	53	M	C20.-	III	Adenocarcinoma
102	P113	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	71	M	C18.7	I	Adenocarcinoma
103	P114	-	Snap-frozen	2	WT	38 G>A	1633 G>A	38 G>A	MSKK	79	M	C20.-	III	Adenocarcinoma
104	P116	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	79	F	C18.0	III	Adenocarcinoma
105	P117	-	Snap-frozen	1	1799 T>A	WT	WT	-	RVS	53	M	C20.-	II	Adenocarcinoma
106	P118	-	Snap-frozen	1	WT	35 G>T	WT	-	RVS	73	M	C20.91	III	Adenocarcinoma
107	P119	-	Snap-frozen	1	WT	35 G>T	WT	-	RVS	75	M	C20.91	II	Adenocarcinoma
108	P120	-	Snap-frozen	2	WT	35 G>A	1633 G>A	35 G>A	RVS	59	M	C20.-	III	Adenocarcinoma
109	P121	-	Snap-frozen	0	WT	WT	WT	-	RVS	43	F	C20.91	I	Adenocarcinoma
110	P122	-	Snap-frozen	0	WT	WT	WT	WT	RVS	47	M	C20.91	III	Adenocarcinoma
111	P123	-	Snap-frozen	1	1799 T>A	WT	WT	-	RVS	55	M	C20.-	III	Adenocarcinoma
112	P124	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	60	M	C20.91	I	Adenocarcinoma
113	P125	-	Snap-frozen	0	WT	WT	WT	WT	RVS	75	F	C20.-	III	Adenocarcinoma
114	P126	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	87	F	C20.-	I	Adenocarcinoma
115	P127	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	MSKK	71	M	C18.7	III	Adenocarcinoma
116	P128	-	Snap-frozen	1	WT	436 G>A	WT	-	MSKK	75	M	C18.2	III	Mucinous Carcinoma
117	P129	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	86	F	C18.0	III	Adenocarcinoma
118	P130	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	67	F	C19.9	III	Adenocarcinoma
119	P25	-	FFPE	0	WT	WT	WT	-	MSKK	74	M	C18.7	III	Adenocarcinoma
120	P131	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	81	F	C18.2	II	Adenocarcinoma
121	P132	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	78	F	C18.7	III	Adenocarcinoma
122	P133	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	MSKK	64	M	C20.9	III	Adenocarcinoma
123	P134	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	MSKK	79	M	C20.-	III	Adenocarcinoma
124	P135	-	Snap-frozen	1	WT	35 G>C	WT	35 G>C	MSKK	71	F	C18.7	III	Adenocarcinoma
125	P136	-	Snap-frozen	1	WT	35 G>A	WT	-	MSKK	76	M	C20.-	II	Adenocarcinoma
126	P137	-	Snap-frozen	1	1799 T>A	WT	WT	WT	MSKK	84	F	C18.2	III	Adenocarcinoma
127	P138	-	Snap-frozen	1	WT	WT	3140 A>G	WT	MSKK	53	F	C18.5	III	Adenocarcinoma

128	P139	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	85	M	C20.-	II	Adenocarcinoma
129	P140	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	66	M	C18.3	III	Adenocarcinoma
130	P141	-	Snap-frozen	1	WT	WT	1624 G>A	WT	MSKK	72	M	C18.6	I	Adenocarcinoma
131	P142	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	60	M	C18.7	III	Adenocarcinoma
132	P143	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	67	M	C18.0	III	Adenocarcinoma
133	P145	-	FFPE	0	WT	WT	WT	-	MSKK	69	F	C18.7	I	Adenocarcinoma
134	P146	-	Snap-frozen	2	WT	35 G>T	1633 G>A	-	MSKK	78	M	C18.6	III	Adenocarcinoma
135	P147	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	66	F	C20.-	III	Adenocarcinoma
136	P148	-	FFPE	0	WT	WT	WT	-	MSKK	79	M	C18.7	I	Adenocarcinoma
137	P149	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	MSKK	82	F	C18.4	III	Adenocarcinoma
138	P8	-	FFPE	2	WT	35 G>A	3140 A>G	NA	MSKK	75	M	C18.4	II	Adenocarcinoma
139	P154	-	Snap-frozen	0	WT	WT	WT	-	MSKK	67	M	C18.7	II	Adenocarcinoma
140	P155	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	68	F	C18.7	II	Adenocarcinoma
141	P150	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	62	M	C18.7	I	Adenocarcinoma
142	P144	-	FFPE	0	WT	WT	WT	WT	MSKK	74	M	C18.0	I	Synchronous CRC
143	P152	-	FFPE	1	WT	38 G>A	WT	-	MSKK	70	M	C20.-	III	Rest after neoadj.th.
144	P153	-	FFPE	0	WT	WT	WT	-	MSKK	68	M	C20.-	I	Adenocarcinoma
145	P157	-	Snap-frozen	2	1799 T>A	WT	1633 G>A	WT	MSKK	67	F	C20.-	III	Adenocarcinoma
146	P158	-	Snap-frozen	0	WT	WT	WT	-	MSKK	48	F	C20.-	III	Adenocarcinoma
147	P159	-	FFPE	0	WT	WT	WT	-	MSKK	63	M	C20.-	III	Adenocarcinoma
148	P160	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	MSKK	46	M	C18.4	III	Adenocarcinoma
149	P161	-	Snap-frozen	0	WT	WT	WT	-	RVS	53	F	C19.9	II	Adenocarcinoma
150	P162	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	72	M	C18.7	II	Adenocarcinoma
151	P163	-	Snap-frozen	0	WT	WT	WT	-	RVS	82	M	C18.7	II	Adenocarcinoma
152	P164	-	Snap-frozen	0	WT	WT	WT	WT	RVS	65	M	C18.9	II	Adenocarcinoma
153	P165	-	Snap-frozen	0	WT	WT	WT	WT	RVS	52	M	C18.7	II	Adenocarcinoma
154	P166	-	Snap-frozen	0	WT	WT	WT	WT	RVS	61	F	C18.0	I	Adenocarcinoma
155	P167	-	Snap-frozen	0	WT	WT	WT	WT	RVS	66	F	C19.-	III	Adenocarcinoma
156	P168	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	RVS	84	F	C18.2	I	Adenocarcinoma
157	P169	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	64	M	C18.0	I	Adenocarcinoma
158	P170	-	Snap-frozen	0	WT	WT	WT	WT	RVS	71	M	C20.-	II	Adenocarcinoma
159	P171	-	Snap-frozen	1	WT	38 G>A	WT	-	RVS	74	F	C18.2	III	Adenocarcinoma
160	P172	-	Snap-frozen	1	WT	WT	3140 A>G; 1633 G>A	WT	RVS	69	M	C20.-	II	Adenocarcinoma
161	P76	-	FFPE	1	WT	WT	3140 A>G	-	MSKK	69	M	C20.-	II	Rest after neoadj.th.

162	P151	-	Snap-frozen	1	1799 T>A	WT	WT	WT	MSKK	76	F	C18.2	II	Adenocarcinoma
163	P173	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	63	F	C18.6	I	Adenocarcinoma
164	P176	-	Snap-frozen	1	WT	WT	3140 A>G	WT	MSKK	64	M	C20.-	II	Adenocarcinoma
165	P178	-	Snap-frozen	0	WT	WT	WT	-	MSKK	55	M	C20.9	III	Adenocarcinoma
166	P179	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	MSKK	67	M	C18.0	III	Adenocarcinoma
167	P180	-	Snap-frozen	2	WT	34 G>T	1624 G>A	-	MSKK	41	F	C18.7	IV	Adenocarcinoma
168	P181	-	FFPE	0	WT	WT	WT	WT	MSKK	62	M	C20.-	III	Adenocarcinoma
169	P182	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	MSKK	71	F	C20.-	III	Adenocarcinoma
170	P184	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	49	F	C18.7	III	Adenocarcinoma
171	P115	-	FFPE	0	WT	WT	WT	-	MSKK	56	F	C18.2	II	Adenocarcinoma
172	P185	-	FFPE	1	WT	34 G>A	WT	34 G>A	MSKK	63	M	C20.-	III	Adenocarcinoma
173	P183	-	Snap-frozen	1	1799 T>A	WT	WT	-	MSKK	84	F	C18.0	III	Adenocarcinoma
174	P177	-	FFPE	1	WT	35 G>T	WT	-	MSKK	67	F	C18.7	III	Mixed Differentiation
175	P187	-	Snap-frozen	1	WT	436 G>A	WT	WT	MSKK	64	M	C19.-	III	Adenocarcinoma
176	P186	-	FFPE	0	WT	WT	WT	WT	MSKK	71	M	C18.0	III	Mucinous Carcinoma
177	P188	-	FFPE	2	WT	35 G>C	3140 A>G	-	MSKK	79	M	C18.7	II	Mixed Diff.with Mucin
178	P189	-	FFPE	0	WT	WT	WT	-	MSKK	81	M	C20.-	II	Adenocarcinoma
179	P190	-	FFPE	1	WT	38 G>A	WT	-	MSKK	83	M	C18.3	II	Adenocarcinoma
180	P191	-	FFPE	0	WT	WT	WT	NA	MSKK	71	M	C20.-	III	Adenocarcinoma
181	P192	-	FFPE	0	WT	WT	WT	-	MSKK	71	F	C18.7	II	Adenocarcinoma
182	P193	-	FFPE	0	WT	WT	WT	-	MSKK	76	F	C18.2	II	Mucinous Carcinoma
183	P194	-	FFPE	2	WT	38 G>A	1624 G>A	-	MSKK	76	M	C18.7	III	Synchronous CRC
184	P196	-	FFPE	2	WT	35 G>T	1633 G>A	-	MSKK	65	M	C20.-	I	Adenocarcinoma
185	P195	-	Snap-frozen	2	WT	34 G>T	1633 G>A	34 G>T	MSKK	66	M	C20.-	III	Adenocarcinoma
186	P197	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	MSKK	55	M	C18.5	III	Adenocarcinoma
187	P198	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	MSKK	80	F	C20.-	III	Adenocarcinoma
188	P201	-	FFPE	0	WT	WT	WT	-	MSKK	46	F	C18.5	IV	Adenosquamous carcinoma
189	P202	-	FFPE	0	WT	WT	WT	WT	MSKK	56	F	C18.2	III	Mucinous Carcinoma
190	P43	-	Snap-frozen	0	WT	WT	WT	WT	RVS	75	M	C18.6	I	Adenocarcinoma
191	P58	-	Snap-frozen	0	WT	WT	WT	WT	RVS	54	M	C18.3	III	Adenocarcinoma
192	P61	-	Snap-frozen	0	WT	WT	WT	-	RVS	54	M	C18.5	II	Adenocarcinoma
193	P64	-	Snap-frozen	0	WT	WT	WT	WT	RVS	70	M	C20.91	II	Adenocarcinoma
194	P69	-	Snap-frozen	0	WT	WT	WT	-	RVS	52	M	C20.-	III	Adenocarcinoma
195	P175	-	FFPE	0	WT	WT	WT	-	MSKK	74	M	C18.7	II	Unusual Differentiation

196	P174	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C18.6	II	Adenocarcinoma
197	P204	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C18.2	III	Mixed Diff.with Mucin
198	P205	-	FFPE	0	WT	WT	WT	-	MSKK	76	M	C18.3	IV	Adenocarcinoma
199	P206	-	FFPE	0	WT	WT	WT	-	MSKK	67	F	C18.2	II	Adenocarcinoma
200	P207	-	FFPE	0	WT	WT	WT	-	MSKK	36	F	C18.7	II	Adenocarcinoma
201	P208	-	Snap-frozen	1	WT	34 G>T	WT	34 G>T	MSKK	75	F	C20.-	III	Adenocarcinoma
202	P209	-	FFPE	0	WT	WT	WT	-	MSKK	83	F	C19.-	II	Adenocarcinoma
203	P210	-	Snap-frozen	2	WT	35 G>T	3140 A>G	35 G>T	MSKK	69	F	C19.-	III	Adenocarcinoma
204	P211	-	FFPE	0	WT	WT	WT	-	MSKK	54	F	C18.6	II	Adenocarcinoma
205	P199	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C20.9	II	Rest after neoadj.th.
206	P212	-	Snap-frozen	1	WT	35 G>A	WT	-	RVS	75	M	C20.-	II	Adenocarcinoma
207	P213	-	Snap-frozen	2	WT	35 G>A	1633 G>A	NA	RVS	70	M	C20.-	IV	Adenocarcinoma
208	P214	-	Snap-frozen	2	WT	35 G>A	1633 G>A	NA	RVS	69	F	C18.7	I	Adenocarcinoma
209	P215	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	78	-	C18.7	II	Adenocarcinoma
210	P216	-	Snap-frozen	0	WT	WT	WT	WT	RVS	79	F	C18.0	III	Adenocarcinoma
211	P217	-	Snap-frozen	0	WT	WT	WT	-	RVS	-	F	C18.6	II	Adenocarcinoma
212	P218	-	Snap-frozen	1	WT	WT	3140 A>G	WT	RVS	63	M	C18.7	III	Adenocarcinoma
213	P219	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	71	F	C18.0	III	Adenocarcinoma
214	P221	-	FFPE	1	WT	436 G>A	WT	-	MSKK	77	M	C18.4	III	Mucinous Carcinoma
215	P222	-	Snap-frozen	0	WT	WT	WT	-	MSKK	57	M	C18.7	III	Adenocarcinoma
216	P226	-	FFPE	1	WT	34 G>T	WT	WT	MSKK	80	M	C20.-	II	Adenocarcinoma
217	P227	-	FFPE	0	WT	WT	WT	-	MSKK	69	F	C20.9	IV	Adenocarcinoma
218	P228	-	Snap-frozen	1	1799 T>A	WT	WT	WT	MSKK	86	F	C18.2	III	Mucinous Carcinoma
219	P220	-	FFPE	1	WT	38 G>A	WT	-	MSKK	77	M	C20.-	III	Mixed Diff.with Mucin
220	P229	-	FFPE	0	WT	WT	WT	-	MSKK	58	M	C18.0	III	Mixed Diff.with Mucin
221	P230	-	FFPE	1	WT	38 G>A	WT	-	MSKK	84	F	C20.-	II	Adenocarcinoma
222	P231	-	FFPE	0	WT	WT	WT	WT	MSKK	56	F	C20.-	III	Rest after neoadj.th.
223	P203	-	Snap-frozen	1	WT	WT	3140 A>G	-	MSKK	79	M	C18.7	III	Adenocarcinoma
224	P232	-	Snap-frozen	1	WT	35 G>C	WT	35 G>C	MSKK	70	F	C18.0	III	Adenocarcinoma
225	P233	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	66	M	C18.6	III	Adenocarcinoma
226	P234	-	FFPE	0	WT	WT	WT	-	MSKK	56	M	C19.9	III	Rest after neoadj.th.
227	P235	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	73	F	C18.7	III	Adenocarcinoma
228	P236	-	FFPE	0	WT	WT	WT	-	MSKK	83	M	C20.-	II	Adenocarcinoma
229	P237	-	FFPE	0	WT	WT	WT	-	MSKK	51	M	C19.-	III	Adenocarcinoma

230	P238	-	FFPE	1	WT	35 G>T	WT	-	MSKK	65	M	C20.-	I	Rest after neoadj.th.
231	P239	-	FFPE	0	WT	WT	WT	-	MSKK	55	M	C20.-	II	Rest after neoadj.th.
232	P240	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	82	M	C18.3	III	Synchronous CRC
233	P241	-	FFPE	1	WT	35 G>A	WT	-	MSKK	51	F	C18.2	I	Adenocarcinoma
234	P242	-	FFPE	0	WT	WT	WT	-	MSKK	73	F	C18.0	I	Mixed Diff.with Mucin
235	P243	-	Snap-frozen	1	WT	34 G>A	WT	-	MSKK	81	F	C20.-	III	Adenocarcinoma
236	P244	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	81	F	C18.3	II	Medullary Differentitation
237	P247	-	Snap-frozen	2	WT	35 G>A	1633 G>A	35 G>A	MSKK	66	M	C18.4	III	Adenocarcinoma
238	P248	-	FFPE	2	WT	38 G>A	1633 G>A	38 G>A	MSKK	66	M	C18.2	IV	Mixed Diff.with Mucin
239	P249	-	FFPE	1	WT	35 G>T	WT	-	MSKK	80	M	C18.3	IV	Mucinous Carcinoma
240	P254	-	FFPE	1	WT	35 G>T	WT	-	MSKK	61	M	C18.0	I	Mixed Diff.with Mucin
241	P255	-	FFPE	0	WT	WT	WT	-	MSKK	68	F	C20.-	III	Adenocarcinoma
242	P258	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	81	F	C18.4	I	Mixed Diff.with Mucin
243	P259	-	Snap-frozen	1	WT	WT	3140 A>G	WT	RVS	64	F	c18.7	II	Adenocarcinoma
244	P260	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	60	M	C18.9	I	Adenocarcinoma
245	P261	-	Snap-frozen	0	WT	WT	WT	WT	RVS	52	M	C18.9	II	Adenocarcinoma
246	P262	-	Snap-frozen	0	WT	WT	WT	-	RVS	71	F	C20.-	II	Adenocarcinoma
247	P263	-	Snap-frozen	1	1799 T>A	WT	WT	-	RVS	60	M	C20.-	III	Adenocarcinoma
248	P264	-	Snap-frozen	2	WT	436 G>A	1633 G>A	WT	RVS	74	M	C18.7	II	Adenocarcinoma
249	P265	-	Snap-frozen	1	WT	35 G>T	WT	-	RVS	70	F	C18.0	III	Adenocarcinoma
250	P266	-	Snap-frozen	2	WT	35 G>A	1633 G>A	35 G>A	RVS	78	F	C18.3	II	Adenocarcinoma
251	P267	-	Snap-frozen	1	WT	WT	3140 A>G; 1633 G>A	WT	RVS	65	F	C18.7	II	Adenocarcinoma
252	P268	-	Snap-frozen	1	WT	35 G>A	WT	-	RVS	76	-	C18.9	I	Adenocarcinoma
253	P269	-	Snap-frozen	0	WT	WT	WT	WT	RVS	34	M	C18.7	II	Adenocarcinoma
254	P270	-	Snap-frozen	2	WT	34 G>A	1633 G>A	34 G>A	RVS	60	M	C20.-	II	Adenocarcinoma
255	P271	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	73	M	C20.-	I	Adenocarcinoma
256	P272	-	Snap-frozen	1	WT	WT	1633 G>A	-	RVS	70	F	C18.9	III	Adenocarcinoma
257	P273	-	Snap-frozen	1	WT	34 G>A	WT	34 G>A	RVS	76	F	C20.-	I	Adenocarcinoma
258	P274	-	Snap-frozen	2	WT	38 G>A	1633 G>A	38 G>A	RVS	85	M	C18.4	II	Adenocarcinoma
259	P275	-	Snap-frozen	2	WT	35 G>T	1633 G>A	35 G>T	RVS	59	M	C18.2	I	Adenocarcinoma
260	P276	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	46	F	C20.-	III	Adenocarcinoma
261	P277	-	Snap-frozen	1	WT	436 G>A	WT	WT	RVS	64	M	C18.0	I	Adenocarcinoma
262	P278	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	41	-	C20.9	III	Adenocarcinoma
263	P279	-	Snap-frozen	0	WT	WT	WT	WT	RVS	59	-	C20.-	III	Adenocarcinoma

264	P280	-	Snap-frozen	0	WT	WT	WT	WT	RVS	67	M	C18.2	I	Adenocarcinoma
265	P281	-	Snap-frozen	1	WT	WT	3140 A>G	WT	RVS	58	M	C20.-	III	Adenocarcinoma
266	P282	-	FFPE	0	WT	WT	WT	WT	MSKK	80	M	C18.5	II	Adenocarcinoma
267	P283	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	MSKK	68	M	C18.2	III	Mucinous Carcinoma
268	P284	-	Snap-frozen	0	WT	WT	WT	-	MSKK	67	M	C20.-	III	Adenocarcinoma
269	P287	-	FFPE	0	WT	WT	WT	NA	MSKK	69	M	C20.-	I	Rest after neoadj.th.
270	P250	-	FFPE	1	WT	35 G>A	WT	-	MSKK	78	F	C18.7	I	Adenocarcinoma
271	P251	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	C18.7	II	Adenocarcinoma
272	P252	-	FFPE	0	WT	WT	WT	-	MSKK	64	M	C18.7	III	Adenocarcinoma
273	P253	-	FFPE	0	WT	WT	WT	-	MSKK	79	M	C18.0	II	Adenocarcinoma
274	P288	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	61	F	C18.0	IV	Adenocarcinoma
275	P289	-	FFPE	0	WT	WT	WT	-	MSKK	70	F	C18.3	III	Undifferentiated
276	P291	-	FFPE	1	WT	35 G>A	WT	-	MSKK	57	F	C18.5	III	Adenocarcinoma
277	P292	-	FFPE	0	WT	WT	WT	-	MSKK	63	F	C18.7	II	Adenocarcinoma
278	P293	-	FFPE	2	WT	38 G>A	1633 G>A	-	MSKK	83	F	C18.0	III	Adenocarcinoma
279	P294	-	FFPE	0	WT	WT	WT	-	MSKK	75	M	C18.0	II	Adenocarcinoma
280	P295	-	FFPE	1	WT	35 G>A	WT	NA	MSKK	75	M	C19.9	III	Mucinous Carcinoma
281	P296	-	FFPE	1	WT	35 G>A	WT	-	MSKK	67	M	C18.0	I	Mucinous Carcinoma
282	P297	-	FFPE	1	WT	38 G>A	WT	-	MSKK	37	M	C19.9	II	Adenocarcinoma
283	P225	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C18.7	IV	Adenocarcinoma
284	P223	-	FFPE	1	WT	38 G>A	WT	-	MSKK	48	M	C18.2	III	Adenocarcinoma
285	P224	-	FFPE	2	1799 T>A	38 G>A	WT	-	MSKK	87	F	C18.0	II	Mixed Diff.with Mucin
286	P19	-	FFPE	0	WT	WT	WT	-	MSKK	68	M	C18.6	III	Adenocarcinoma
287	P88	-	FFPE	2	WT	34 G>T	1624 G>A	-	MSKK	68	F	C18.6	II	Adenocarcinoma
288	P298	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C18.7	IV	Adenocarcinoma
289	P299	-	FFPE	1	WT	34 G>T	WT	-	MSKK	65	M	C18.7	II	Adenocarcinoma
290	P300	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	67	F	C20.9	III	Adenocarcinoma
291	P301	-	FFPE	0	WT	WT	WT	-	MSKK	74	M	C18.7	IV	Adenocarcinoma
292	P302	-	FFPE	1	WT	35 G>A	WT	-	MSKK	76	M	C20.9	II	Adenocarcinoma
293	P290	-	FFPE	1	WT	35 G>A	WT	NA	MSKK	68	F	C20.-	III	Adenocarcinoma
294	P303	-	FFPE	1	WT	436 G>A	WT	-	MSKK	88	M	C20.-	I	Adenocarcinoma
295	P304	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	67	M	C18.7	III	Synchronous CRC
296	P305	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	76	M	C18.2	III	Adenocarcinoma
297	P286	-	FFPE	3	1799 T>A	38 G>A	1633 G>A	WT	MSKK	69	F	C18.7	III	Adenocarcinoma

298	P307	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	75	F	C18.7	III	Mucinous Carcinoma
299	P308	-	FFPE	1	WT	35 G>A	WT	-	MSKK	68	F	C18.2	II	Mixed Diff.with Mucin
300	P309	-	FFPE	1	WT	35 G>T	WT	-	MSKK	77	M	C18.4	II	Adenocarcinoma
301	P310	-	FFPE	1	WT	34 G>A	WT	-	MSKK	64	M	C18.6	III	Adenocarcinoma
302	P311	-	FFPE	2	WT	35 G>A	3140 A>G	-	MSKK	48	F	C18.3	II	Mucinous Carcinoma
303	P312	-	FFPE	0	WT	WT	WT	-	MSKK	85	F	C18.7	III	Adenocarcinoma
304	P313	-	FFPE	0	WT	WT	WT	-	MSKK	84	F	C19.-	I	Adenocarcinoma
305	P314	-	FFPE	2	WT	35 G>T	1633 G>A	-	MSKK	75	M	C18.0	III	Adenocarcinoma
306	P315	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C18.7	III	Adenocarcinoma
307	P316	-	FFPE	1	WT	35 G>T	WT	-	MSKK	65	M	C18.7	II	Mixed Diff.with Mucin
308	P317	-	FFPE	2	WT	34 G>T	3140 A>G	-	MSKK	76	M	C18.2	IV	Adenocarcinoma
309	P318	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	C20.-	II	Adenocarcinoma
310	P319	-	FFPE	1	WT	WT	3140 A>G	-	MSKK	82	F	C18.2	III	Adenocarcinoma
311	P320	-	FFPE	0	WT	WT	WT	-	MSKK	81	F	C18.2	II	Mucinous Carcinoma
312	P321	-	FFPE	1	WT	35 G>A	WT	-	MSKK	70	M	C18.7	III	Adenocarcinoma
313	P322	-	FFPE	1	WT	35 G>A	WT	-	MSKK	62	M	C20.-	III	Rest after neoadj.th.
314	P323	-	FFPE	0	WT	WT	WT	NA	MSKK	76	M	C20.9	III	Adenocarcinoma
315	P324	-	FFPE	0	WT	WT	WT	-	MSKK	84	F	C19.-	II	Adenocarcinoma
316	P325	-	FFPE	1	WT	38 G>A	WT	-	MSKK	64	F	C20.-	III	Rest after neoadj.th.
317	P326	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C18.7	III	Adenocarcinoma
318	P328	-	FFPE	2	WT	35 G>A	3140 A>G	-	MSKK	71	F	C19.-	II	Rest after neoadj.th.
319	P329	-	Snap-frozen	1	1799 T>A	WT	WT	WT	MSKK	79	M	C18.0	III	Adenocarcinoma
320	P331	-	Snap-frozen	1	WT	35 G>A	WT	-	RVS	69	F	C20.-	III	Adenocarcinoma
321	P332	-	Snap-frozen	0	WT	WT	WT	WT	RVS	65	M	C18.3	II	Adenocarcinoma
322	P333	-	Snap-frozen	0	WT	WT	WT	WT	RVS	59	M	C18.9	II	Adenocarcinoma
323	P334	-	Snap-frozen	0	WT	WT	WT	WT	RVS	67	F	C20.-	II	Adenocarcinoma
324	P335	-	Snap-frozen	0	WT	WT	WT	WT	RVS	67	-	C18.7	I	Adenocarcinoma
325	P336	-	Snap-frozen	2	WT	436 G>A	1633 G>A	WT	RVS	77	F	C20.-	III	Adenocarcinoma
326	P337	-	Snap-frozen	1	WT	35 G>A	WT	-	RVS	64	M	C18.0	II	Adenocarcinoma
327	P338	-	Snap-frozen	1	WT	35 G>C	WT	35 G>C	RVS	43	F	C20.-	III	Adenocarcinoma
328	P339	-	Snap-frozen	0	WT	WT	WT	WT	RVS	57	M	C19.-	II	Adenocarcinoma
329	P340	-	Snap-frozen	0	WT	WT	WT	WT	RVS	46	M	C18.7	I	Adenocarcinoma
330	P341	-	Snap-frozen	0	WT	WT	WT	WT	RVS	61	F	C20.-	III	Adenocarcinoma
331	P342	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	71	M	C18.2	III	Adenocarcinoma

332	P343	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	75	F	C18.0	II	Adenocarcinoma
333	P344	-	Snap-frozen	0	WT	WT	WT	-	RVS	66	M	C18.9	III	Adenocarcinoma
334	P345	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	60	-	C20.-	III	Adenocarcinoma
335	P346	-	Snap-frozen	0	WT	WT	WT	WT	RVS	60	M	C18.2	III	Adenocarcinoma
336	P347	-	Snap-frozen	0	WT	WT	WT	WT	RVS	40	F	C18.9	II	Adenocarcinoma
337	P348	-	Snap-frozen	0	WT	WT	WT	WT	RVS	-	-	C20.-	II	Adenocarcinoma
338	P349	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	66	M	C18.0	III	Mucinous Carcinoma
339	P350	-	Snap-frozen	1	WT	38 G>A	WT	-	RVS	68	M	C20.-	I	Adenocarcinoma
340	P351	-	Snap-frozen	0	WT	WT	WT	WT	RVS	74	M	C18.0	II	Adenocarcinoma
341	P352	-	Snap-frozen	1	WT	35 G>T	WT	-	RVS	69	M	C18.9	I	Adenocarcinoma
342	P353	-	Snap-frozen	1	WT	34 G>T	WT	34 G>T	RVS	77	M	C18.0	II	Adenocarcinoma
343	P354	-	Snap-frozen	0	WT	WT	WT	WT	RVS	59	-	C20.-	III	Adenocarcinoma
344	P355	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	C18.2	III	Adenocarcinoma
345	P356	-	FFPE	0	WT	WT	WT	-	MSKK	76	M	C20.9	III	Rest after neoadj.th.
346	P306	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	83	F	C18.4	I	Mucinous Carcinoma
347	P357	-	FFPE	1	WT	38 G>A	WT	38 G>A	MSKK	73	M	C18.7	I	Adenocarcinoma
348	P359	-	FFPE	0	WT	WT	WT	-	MSKK	63	M	C20.-	III	Rest after neoadj.th.
349	P360	-	FFPE	1	WT	WT	1624 G>A	-	MSKK	69	F	C18.0	II	Mucinous Carcinoma
350	P361	-	FFPE	0	WT	WT	WT	-	MSKK	76	F	C18.0	II	Adenocarcinoma
351	P362	-	FFPE	1	WT	35 G>A	WT	-	MSKK	82	F	C18.4	II	Adenocarcinoma
352	P363	-	FFPE	0	WT	WT	WT	WT	MSKK	72	M	C18.7	III	Adenocarcinoma
353	P364	-	FFPE	0	WT	WT	WT	WT	MSKK	77	M	C18.3	III	Adenocarcinoma
354	P156	-	FFPE	1	WT	38 G>A	WT	-	MSKK	67	M	C20.-	II	Adenocarcinoma
355	P367	-	FFPE	0	WT	WT	WT	-	MSKK	65	M	C20.-	I	Rest after neoadj.th.
356	P368	-	Snap-frozen	1	WT	436 G>A	WT	WT	MSKK	87	F	C18.7	III	Adenocarcinoma
357	P369	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	71	F	C18.4	III	Adenocarcinoma
358	P370	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	52	F	C18.4	I	Tumour in Adenoma
359	P372	-	FFPE	0	WT	WT	WT	-	MSKK	63	M	C20.-	II	Adenocarcinoma
360	P373	-	FFPE	1	WT	38 G>A	WT	-	MSKK	57	M	C18.2	II	Adenocarcinoma
361	P374	-	FFPE	0	WT	WT	WT	-	MSKK	79	F	C18.7	III	Adenocarcinoma
362	P375	-	FFPE	1	WT	35 G>A	WT	-	MSKK	79	F	C18.0	II	Mucinous Carcinoma
363	P376	-	FFPE	1	WT	35 G>A	WT	-	MSKK	83	F	C18.7	III	Adenocarcinoma
364	P377	-	FFPE	0	WT	WT	WT	WT	MSKK	86	F	C18.7	II	Adenocarcinoma
365	P358	-	Snap-frozen	1	1799 T>A	WT	WT	WT	MSKK	66	F	C18.4	III	Tumour in Adenoma

366	P379	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	52	M	C18.2	III	Neuroendocrine Diff.
367	P381	-	FFPE	1	WT	35 G>A	WT	-	MSKK	68	M	C18.0	III	Mucinous Carcinoma
368	P382	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	82	F	C18.6	II	Mixed Diff.with Mucin
369	P383	-	FFPE	1	WT	35 G>A	WT	-	MSKK	87	M	C18.6	II	Adenocarcinoma
370	P365	-	FFPE	1	WT	35 G>A	WT	-	MSKK	73	F	C20.9	III	Mixed Diff.with Mucin
371	P366	-	FFPE	1	WT	35 G>T	WT	-	MSKK	79	F	C18.0	II	Adenocarcinoma
372	P384	-	FFPE	2	WT	35 G>T	3140 A>G	-	MSKK	75	M	C18.4	II	Adenocarcinoma
373	P385	-	FFPE	1	WT	34 G>A	WT	-	MSKK	80	F	C18.0	III	Adenocarcinoma
374	P386	-	FFPE	2	1799 T>A	WT	1633 G>A	-	MSKK	62	F	C18.2	III	Mucinous Carcinoma
375	P387	-	FFPE	0	WT	WT	WT	-	MSKK	48	M	C19.9	IV	Adenocarcinoma
376	P388	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	55	F	C19.9	III	Adenocarcinoma
377	P389	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C20.9	III	Adenocarcinoma
378	P390	-	FFPE	1	WT	35 G>C	WT	-	MSKK	73	M	C20.-	II	Adenocarcinoma
379	P391	-	FFPE	0	WT	WT	WT	-	MSKK	62	M	C18.0	III	Adenocarcinoma
380	P330	-	FFPE	1	WT	35 G>T	WT	-	MSKK	65	M	C18.7	II	Adenocarcinoma
381	P394	-	FFPE	0	WT	WT	WT	-	MSKK	76	F	C18.2	II	Adenocarcinoma
382	P395	-	FFPE	0	WT	WT	WT	NA	MSKK	75	M	C20.-	II	Adenocarcinoma
383	P396	-	FFPE	1	WT	38 G>A	WT	-	MSKK	78	F	C19.-	III	Unusual Differentiation
384	P397	-	FFPE	0	WT	WT	WT	WT	MSKK	67	M	C20.-	I	Neuroendocrine Diff.
385	P398	-	FFPE	0	WT	WT	WT	-	MSKK	82	F	C18.6	II	Adenocarcinoma
386	P399	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C18.7	IV	Adenocarcinoma
387	P400	-	FFPE	0	WT	WT	WT	-	MSKK	77	F	C18.2	II	Mucinous Carcinoma
388	P401	-	FFPE	2	WT	35 G>T	1633 G>A	35 G>T	MSKK	65	M	C18.2	III	Mucinous Carcinoma
389	P402	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	MSKK	69	M	C18.7	III	Adenocarcinoma
390	P403	-	FFPE	0	WT	WT	WT	-	MSKK	48	M	C19.9	IV	Adenocarcinoma
391	P404	-	FFPE	1	WT	35 G>A	WT	-	MSKK	66	M	C18.0	III	Tumour in Adenoma
392	P378	-	FFPE	0	WT	WT	WT	-	MSKK	68	M	C18.2	II	Mucinous Carcinoma
393	P406	-	FFPE	0	WT	WT	WT	WT	MSKK	61	F	C18.5	III	Adenocarcinoma
394	P409	-	Snap-frozen	0	WT	WT	WT	-	MSKK	64	M	C20.9	III	Adenocarcinoma
395	P410	-	FFPE	0	WT	WT	WT	-	MSKK	87	F	C20.-	II	Adenocarcinoma
396	P411	-	FFPE	1	WT	34 G>A	WT	-	MSKK	66	M	C20.-	I	Adenocarcinoma
397	P380	-	FFPE	0	WT	WT	WT	-	MSKK	63	F	C20.-	I	Adenocarcinoma
398	P414	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	80	F	C18.3	III	Adenocarcinoma
399	P415	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	66	F	C18.7	III	Adenocarcinoma

400	P392	-	FFPE	2	1799 T>A	WT	1633 G>A	-	MSKK	65	F	C18.4	I	Mucinous Carcinoma
401	P393	-	FFPE	0	WT	WT	WT	-	MSKK	63	M	C20.-	III	Adenocarcinoma
402	P416	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	65	M	C18.0	I	Mixed Diff.with Mucin
403	P422	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	MSKK	77	M	C19.9	III	Adenocarcinoma
404	P424	-	FFPE	1	WT	34 G>A	WT	-	MSKK	76	M	C18.7	IV	Synchronous CRC
405	P412	-	FFPE	2	WT	35 G>C	1633 G>A	-	MSKK	84	M	C18.3	II	Adenocarcinoma
406	P413	-	FFPE	0	WT	WT	WT	WT	MSKK	80	M	C20.-	I	Adenocarcinoma
407	P425	-	FFPE	0	WT	WT	WT	-	MSKK	58	M	C20.-	III	Unusual Differentiation
408	P426	-	FFPE	1	1799 T>A	WT	WT	WT	MSKK	75	M	C18.4	III	Unusual Differentiation
409	P427	-	FFPE	0	WT	WT	WT	-	MSKK	63	M	C20.-	III	Mucinous Carcinoma
410	P428	-	FFPE	2	WT	35 G>T	1624 G>A	35 G>T	MSKK	62	M	C18.0	II	Mixed Diff.with Mucin
411	P419	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	82	F	C18.7	IV	Mixed Diff.with Mucin
412	P420	-	FFPE	1	WT	WT	1633 G>A	-	MSKK	75	M	C18.7	II	Mixed Diff.with Mucin
413	P429	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	72	F	C18.0	III	Unusual Differentiation
414	P430	-	FFPE	0	WT	WT	WT	-	MSKK	63	F	C19.-	I	Tumour in Adenoma
415	P431	-	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	52	M	C18.0	III	Adenocarcinoma
416	P432	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	82	M	C18.2	II	Adenocarcinoma
417	P433	-	FFPE	1	1799 T>A	WT	WT	WT	MSKK	65	M	C18.4	III	Adenocarcinoma
418	P436	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	67	F	C18.0	II	Mixed Diff.with Mucin
419	P437	-	FFPE	0	WT	WT	WT	-	MSKK	75	M	C18.4	IV	Adenocarcinoma
420	P438	-	FFPE	1	WT	35 G>A	WT	-	MSKK	82	M	C18.2	III	Mucinous Carcinoma
421	P439	-	FFPE	1	WT	35 G>T	WT	-	MSKK	58	M	C20.-	III	Adenocarcinoma
422	P440	-	FFPE	0	WT	WT	WT	-	MSKK	82	F	C18.7	I	Adenocarcinoma
423	P441	-	FFPE	0	WT	WT	WT	-	MSKK	63	M	C18.9	II	Adenocarcinoma
424	P442	-	FFPE	1	WT	35 G>A	WT	-	MSKK	82	F	C20.-	I	Adenocarcinoma
425	P443	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C18.2	III	Adenocarcinoma
426	P444	-	FFPE	1	WT	34 G>A	WT	-	MSKK	73	M	C20.-	III	Adenocarcinoma
427	P445	-	FFPE	1	WT	34 G>T	WT	34 G>T	MSKK	58	M	C18.0	III	Adenocarcinoma
428	P446	-	FFPE	1	WT	35 G>T	WT	-	MSKK	77	F	C18.-	III	Synchronous CRC
429	P447	-	FFPE	1	WT	35 G>A	WT	-	MSKK	70	F	C18.0	III	Unusual Differentiation
430	P448	-	FFPE	2	WT	38 G>A	3140 A>G	-	MSKK	73	F	C20.-	III	Rest after neoadj.th.
431	P449	-	FFPE	2	WT	35 G>T	1633 G>A	-	MSKK	77	F	C18.3	IV	Synchronous CRC
432	P451	-	FFPE	0	WT	WT	WT	-	MSKK	80	M	C20.-	I	Adenocarcinoma
433	P452	-	FFPE	0	WT	WT	WT	-	MSKK	61	M	C20.-	I	Adenocarcinoma

434	P453	-	FFPE	2	WT	35 G>A	1624 G>A	-	MSKK	66	M	C18.7	IV	Adenocarcinoma
435	P454	-	FFPE	1	WT	436 G>A	WT	-	MSKK	86	M	C18.0	III	Neuroendocrine Diff.
436	P455	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	68	F	C18.7	I	Adenocarcinoma
437	P456	-	FFPE	1	WT	35 G>T	WT	35 G>T	MSKK	68	M	C18.0	II	Adenocarcinoma
438	P457	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	79	M	C18.7	I	Adenocarcinoma
439	P458	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C20.9	III	Rest after neoadj.th.
440	P459	-	FFPE	0	WT	WT	WT	WT	MSKK	75	M	C20.-	II	Rest after neoadj.th.
441	P462	-	FFPE	0	WT	WT	WT	NA	MSKK	87	F	C18.3	III	Adenocarcinoma
442	P463	-	FFPE	0	WT	WT	WT	-	MSKK	69	F	C18.0	II	Unusual Differentiation
443	P464	-	FFPE	1	WT	38 G>A	WT	-	MSKK	72	F	C18.0	III	Mixed Diff.with Mucin
444	P465	-	FFPE	0	WT	WT	WT	-	MSKK	52	M	C18.7	II	Adenocarcinoma
445	P466	-	FFPE	0	WT	WT	WT	-	MSKK	64	M	C18.7	IV	Neuroendocrine Diff.
446	P467	-	FFPE	1	WT	38 G>A	WT	-	MSKK	70	M	C20.-	III	Rest after neoadj.th.
447	P468	-	FFPE	1	WT	35 G>A	WT	-	MSKK	57	M	C18.6	II	Mucinous Carcinoma
448	P469	-	FFPE	1	WT	35 G>T	WT	-	MSKK	76	M	C20.9	I	Tumour in Adenoma
449	P417	-	FFPE	0	WT	WT	WT	-	MSKK	64	M	C20.-	I	Adenocarcinoma
450	P473	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C18.4	II	Adenocarcinoma
451	P475	-	FFPE	0	WT	WT	WT	-	MSKK	79	M	C18.9	II	Adenocarcinoma
452	P408	-	FFPE	1	WT	35 G>A	WT	-	MSKK	53	M	C18.7	I	Adenocarcinoma
453	P476	-	FFPE	1	WT	WT	1624 G>A	WT	MSKK	67	F	C18.5	I	Adenocarcinoma
454	P477	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C20.-	II	Adenocarcinoma
455	P478	-	FFPE	1	WT	35 G>T	WT	-	MSKK	78	F	C18.6	II	Adenocarcinoma
456	P479	-	FFPE	0	WT	WT	WT	-	MSKK	76	M	C18.2	II	Adenocarcinoma
457	P480	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	79	F	C18.2	III	Adenocarcinoma
458	P434	-	FFPE	2	WT	34 G>C	3140 A>G	-	MSKK	80	F	C19.-	III	Mucinous Carcinoma
459	P435	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	C19.9	II	Adenocarcinoma
460	P481	-	FFPE	0	WT	WT	WT	-	MSKK	64	M	C18.6	II	Adenocarcinoma
461	P482	-	FFPE	1	WT	38 G>A	WT	NA	MSKK	78	M	C18.3	III	Adenocarcinoma
462	P483	-	FFPE	2	WT	35 G>T	3140 A>G	-	MSKK	72	M	C18.2	II	Tumour in Adenoma
463	P484	-	FFPE	0	WT	WT	WT	-	MSKK	79	F	C18.0	III	Synchronous CRC
464	P485	-	FFPE	1	WT	35 G>A	WT	-	MSKK	72	M	C18.7	I	Mucinous Carcinoma
465	P486	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C18.2	III	Unusual Differentiation
466	P487	-	FFPE	0	WT	WT	WT	-	MSKK	75	M	C18.2	I	Adenocarcinoma
467	P489	-	FFPE	0	WT	WT	WT	-	MSKK	56	M	C20.-	IV	Adenocarcinoma

468	P490	-	FFPE	1	WT	WT	1624 G>A	-	MSKK	66	F	C18.7	II	Adenocarcinoma
469	P492	-	FFPE	0	WT	WT	WT	-	MSKK	39	F	C18.7	II	Adenocarcinoma
470	P493	-	FFPE	0	WT	WT	WT	NA	MSKK	64	M	C20.-	IV	Synchronous CRC
471	P495	-	FFPE	0	WT	WT	WT	-	MSKK	60	M	C19.-	I	Adenocarcinoma
472	P496	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C18.7	I	Adenocarcinoma
473	P498	-	FFPE	0	WT	WT	WT	-	MSKK	60	F	C18.7	II	Adenocarcinoma
474	P503	-	FFPE	0	WT	WT	WT	-	MSKK	78	M	C18.3	III	Mixed Differentiation
475	P504	-	FFPE	0	WT	WT	WT	-	MSKK	78	M	C18.7	I	Adenocarcinoma
476	P505	-	FFPE	0	WT	WT	WT	-	MSKK	71	M	C18.7	IV	Adenocarcinoma
477	P494	M1	FFPE	0	WT	WT	WT	-	MSKK	75	F	C18.7	II	Adenocarcinoma
478	P507	-	FFPE	0	WT	WT	WT	-	MSKK	56	F	C20.-	III	Adenocarcinoma
479	P488	-	FFPE	1	WT	35 G>T	WT	-	MSKK	71	M	C20.-	I	Adenocarcinoma
480	P508	-	FFPE	1	WT	WT	1633 G>A	-	MSKK	74	F	C20.9	I	Adenocarcinoma
481	P510	-	FFPE	2	1799 T>A	WT	3140 A>G	-	MSKK	63	M	C18.2	II	Mucinous Carcinoma
482	P512	-	FFPE	0	WT	WT	WT	-	MSKK	56	F	C18.2	III	Adenocarcinoma
483	P513	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	76	M	C18.3	II	Adenocarcinoma
484	P511	-	FFPE	1	WT	38 G>A	WT	-	MSKK	75	F	C18.7	II	Adenocarcinoma
485	P472	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	C18.7	IV	Adenocarcinoma
486	P501	-	FFPE	0	WT	WT	WT	WT	MSKK	72	F	C18.7	I	Adenocarcinoma
487	P502	-	FFPE	1	WT	34 G>T	WT	34 G>T	MSKK	81	M	C18.4	I	Adenocarcinoma
488	P518	-	Snap-frozen	0	WT	WT	WT	WT	MSKK	82	M	C18.2	III	Synchronous CRC
489	P519	-	FFPE	1	WT	35 G>T	WT	-	MSKK	62	M	C20.9	IV	Adenocarcinoma
490	P521	-	FFPE	0	WT	WT	WT	-	MSKK	82	F	C18.7	II	Adenocarcinoma
491	P497	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	C20.9	I	Adenocarcinoma
492	P423	-	FFPE	0	WT	WT	WT	-	MSKK	65	F	C18.4	I	Adenocarcinoma
493	P506	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C18.7	II	Adenocarcinoma
494	P450	-	FFPE	0	WT	WT	WT	-	MSKK	46	F	C18.0	I	Tumour in Adenoma
495	P418	-	FFPE	1	WT	35 G>A	WT	-	MSKK	82	F	C18.3	III	Mucinous Carcinoma
496	P421	-	FFPE	0	WT	WT	WT	WT	MSKK	64	M	C20.-	III	Adenocarcinoma
497	P522	-	FFPE	0	WT	WT	WT	-	MSKK	52	F	C18.7	II	Adenocarcinoma
498	P461	-	FFPE	1	WT	35 G>T	WT	WT	MSKK	63	F	C20.9	II	Rest after neoadj.th.
499	P460	-	FFPE	0	WT	WT	WT	-	MSKK	78	M	C20.-	I	Adenocarcinoma
500	P520	-	FFPE	0	WT	WT	WT	-	MSKK	74	M	C18.7	I	Adenocarcinoma
501	P509	M3	FFPE	1	WT	35 G>C	WT	-	MSKK	56	M	C18.6	IV	Adenocarcinoma

502	P407	-	FFPE	1	WT	38 G>A	WT	-	MSKK	77	F	C20.-	IV	Mixed Diff.with Mucin
503	P524	-	FFPE	1	1799 T>A	WT	WT	WT	MSKK	81	F	C18.4	III	Mucinous Carcinoma
504	P525	M7	FFPE	0	WT	WT	WT	-	MSKK	69	M	C18.7	II	Adenocarcinoma
505	P526	-	FFPE	2	WT	35 G>A	1633 G>A	35 G>A	MSKK	73	F	C20.-	I	Mucinous Carcinoma
506	P527	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	60	F	C18.0	I	Adenocarcinoma
507	P523	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	77	F	C18.0	I	Mucinous Carcinoma
508	P515	-	FFPE	1	WT	34 G>A, 436 G>A	WT	-	MSKK	41	F	C18.4	III	Mucinous Carcinoma
509	P529	-	FFPE	0	WT	WT	WT	-	MSKK	58	F	C18.0	IV	Adenocarcinoma
510	P532	-	FFPE	1	WT	35 G>A	WT	-	MSKK	65	M	C18.5	II	Adenocarcinoma
511	P530	-	FFPE	0	WT	WT	WT	WT	MSKK	76	F	C18.2	III	Medullary Differentitation
512	P531	-	FFPE	1	WT	WT	1633 G>A	-	MSKK	56	M	C18.3	II	Mixed Diff.with Mucin
513	P516	-	FFPE	0	WT	WT	WT	-	MSKK	73	M	C18.7	III	Adenocarcinoma
514	P535	-	FFPE	1	WT	35 G>T	WT	-	MSKK	67	F	C18.7	II	Adenocarcinoma
515	P536	M10	FFPE	1	1799 T>A	WT	WT	-	MSKK	77	M	C18.2	III	Adenocarcinoma
516	P537	-	FFPE	0	WT	WT	WT	-	MSKK	80	F	C18.3	II	Adenocarcinoma
517	P538	-	FFPE	2	1799 T>A	WT	1633 G>A	-	MSKK	57	F	C18.7	III	Adenocarcinoma
518	P539	-	FFPE	1	1799 T>A	WT	WT	WT	MSKK	77	F	C18.2	II	Mixed Diff.with Mucin
519	P540	-	FFPE	0	WT	WT	WT	WT	MSKK	61	M	C18.2	III	Adenocarcinoma
520	P528	-	FFPE	0	WT	WT	WT	-	MSKK	77	F	C20.-	IV	Adenocarcinoma
521	P541	-	FFPE	0	WT	WT	WT	-	MSKK	75	M	C19.-	III	Rest after neoadj.th.
522	P533	-	FFPE	0	WT	WT	WT	-	MSKK	65	F	C18.4	II	Neuroendocrine Diff.
523	P542	-	FFPE	0	WT	WT	WT	-	MSKK	62	M	C18.7	III	Adenocarcinoma
524	P534	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	69	F	C18.2	III	Mixed Diff.with Mucin
525	P544	-	FFPE	0	WT	WT	WT	-	MSKK	68	M	C18.6	II	Adenocarcinoma
526	P545	-	FFPE	1	WT	436 G>A	WT	-	MSKK	70	M	C18.7	II	Adenocarcinoma
527	P554	-	FFPE	0	WT	WT	WT	-	MSKK	62	M	C20.-	III	Synchronous CRC
528	P546	-	FFPE	1	WT	35 G>A	WT	-	MSKK	76	M	C18.7	II	Mixed Diff.with Mucin
529	P547	-	FFPE	1	WT	35 G>T	WT	-	MSKK	73	M	C20.-	III	Rest after neoadj.th.
530	P548	-	FFPE	1	WT	WT	1624 G>A	WT	MSKK	-	-	-	-	Mixed Diff.with Mucin
531	P549	-	FFPE	1	WT	38 G>A	WT	-	MSKK	-	-	-	-	Rest after neoadj.th.
532	P550	-	FFPE	0	WT	WT	WT	-	MSKK	73	M	C18.7	IV	Adenocarcinoma
533	P551	-	FFPE	0	WT	WT	WT	WT	MSKK	67	F	C18.9	III	Adenocarcinoma
534	P517	-	FFPE	1	WT	35 G>T	WT	-	MSKK	70	F	C18.7	IV	Adenocarcinoma
535	P405	-	FFPE	0	WT	WT	WT	-	MSKK	72	F	C18.2	II	Adenocarcinoma

536	P246	-	FFPE	2	WT	35 G>A	1624 G>A	-	MSKK	78	M	C18.2	II	Adenocarcinoma
537	P245	-	FFPE	1	WT	35 G>A	WT	-	MSKK	73	M	C18.5	III	Adenocarcinoma
538	P285	-	FFPE	0	WT	WT	WT	-	MSKK	81	M	C18.2	II	Adenocarcinoma
539	P552	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	c18.0	II	Adenocarcinoma
540	P500	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C18.6	I	Adenocarcinoma
541	P499	-	FFPE	0	WT	WT	WT	-	MSKK	81	F	C18.0	IV	Adenocarcinoma
542	P553	-	FFPE	1	WT	38 G>A	WT	-	MSKK	72	M	C18.2	II	Mixed Diff.with Mucin
543	P555	-	FFPE	0	WT	WT	WT	-	MSKK	47	F	C20.-	II	Adenocarcinoma
544	P556	-	FFPE	0	WT	WT	WT	WT	MSKK	61	M	C20.-	I	Rest after neoadj.th.
545	P557	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C18.6	III	Mucinous Carcinoma
546	P491	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C18.7	II	Adenocarcinoma
547	P558	-	FFPE	1	WT	WT	1633 G>A	-	MSKK	72	M	C18.0	II	Mucinous Carcinoma
548	P559	-	FFPE	0	WT	WT	WT	WT	MSKK	77	F	C18.4	II	Adenocarcinoma
549	P578	-	FFPE	1	WT	35 G>A	WT	-	MSKK	64	M	C18.0	I	Mixed Diff.with Mucin
550	P561	-	FFPE	0	WT	WT	WT	-	MSKK	57	M	C20.-	II	Adenocarcinoma
551	P543	-	FFPE	1	WT	34 G>T	WT	-	MSKK	39	F	C18.7	III	Adenocarcinoma
552	P474	-	FFPE	0	WT	WT	WT	-	MSKK	62	M	C18.7	III	Adenocarcinoma
553	P371	-	FFPE	1	WT	WT	1633 G>A	-	MSKK	44	M	C20.-	II	Rest after neoadj.th.
554	P327	-	FFPE	0	WT	WT	WT	-	MSKK	66	F	C20.-	II	Rest after neoadj.th.
555	P200	-	FFPE	0	WT	WT	WT	WT	MSKK	68	M	C18.2	III	Mucinous Carcinoma
556	P563	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	MSKK	73	M	C18.7	III	Synchronous CRC
557	P564	-	FFPE	0	WT	WT	WT	-	MSKK	65	M	C20.-	-	Adenocarcinoma
558	P565	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	77	F	C18.0	I	Synchronous CRC
559	P566	-	FFPE	1	WT	35 G>A	WT	-	MSKK	75	M	C20.-	II	Adenocarcinoma
560	P568	-	FFPE	1	WT	35 G>T	WT	-	MSKK	77	M	C18.0	II	Adenocarcinoma
561	P569	-	FFPE	1	WT	35 G>A	WT	-	MSKK	80	M	C18.3	II	Adenocarcinoma
562	P570	-	FFPE	0	WT	WT	WT	WT	MSKK	78	F	C20.-	III	Adenocarcinoma
563	P572	-	FFPE	2	WT	38 G>A	3140 A>G	-	MSKK	69	M	C18.4	IV	Adenocarcinoma
564	P573	-	FFPE	2	WT	35 G>A	1624 G>A; 1633 G>A	-	MSKK	84	F	C18.7	III	Synchronous CRC
565	P575	M19	FFPE	2	1799 T>A	WT	3140 A>G	-	MSKK	68	M	C18.2	I	Tumour in Adenoma
566	P576	M20	FFPE	1	1799 T>A	WT	WT	-	MSKK	71	M	C18.7	IV	Adenocarcinoma
567	P577	-	FFPE	0	WT	WT	WT	NA	MSKK	79	M	C19.-	III	Adenocarcinoma
568	P574	M18	FFPE	1	WT	35 G>A	WT	-	MSKK	59	F	C18.7	II	Adenocarcinoma
569	P562	-	FFPE	0	WT	WT	WT	WT	MSKK	77	M	C18.7	III	Adenocarcinoma

570	P582	M21	FFPE	1	1799 T>A	WT	WT	-	MSKK	77	F	C18.0	III	Tumour in Adenoma
571	P584	-	FFPE	1	WT	38 G>A	WT	-	MSKK	65	M	C20.9	I	Adenocarcinoma
572	P585	-	FFPE	0	WT	WT	WT	WT	MSKK	68	M	C20.-	I	Rest after neoadj.th.
573	P586	-	FFPE	2	WT	35 G>A	1624 G>A	-	MSKK	80	M	C18.7	II	Adenocarcinoma
574	P587	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C20.9	I	Tumour in Adenoma
575	P588	-	FFPE	1	WT	35 G>A	WT	-	MSKK	84	F	C18.2	III	Small Cell Carcinoma
576	P589	-	FFPE	0	WT	WT	WT	-	MSKK	74	M	C18.0	I	Synchronous CRC
577	P590	M24	FFPE	1	WT	35 G>T	WT	-	MSKK	66	F	C20.-	III	Adenocarcinoma
578	P591	-	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	74	M	C18.7	I	Adenocarcinoma
579	P592	M25	FFPE	1	WT	34 G>T	WT	-	MSKK	71	F	C18.6	II	Mixed Diff.with Mucin
580	P593	-	FFPE	1	WT	34 G>T	WT	-	MSKK	69	F	C20.-	II	Mucinous Carcinoma
581	P594	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	78	M	C18.2	II	Undifferentiated
582	P571	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	73	F	C18.0	III	Adenocarcinoma
583	P583	M22	FFPE	0	WT	WT	WT	-	MSKK	82	M	C18.7	III	Adenocarcinoma
584	P596	M27	FFPE	1	WT	35 G>A	WT	-	MSKK	56	F	C20.-	III	Adenocarcinoma
585	P599	-	FFPE	2	WT	34 G>A	3140 A>G	-	MSKK	68	F	C19.9	III	Adenocarcinoma
586	P580	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	83	F	-	I	Neuroendocrine Diff.
587	P579	-	FFPE	0	WT	WT	WT	-	MSKK	66	F	C19.-	II	Adenocarcinoma
588	P601	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	75	F	C18.2	III	Adenocarcinoma
589	P602	-	FFPE	0	WT	WT	WT	WT	MSKK	65	M	C20.-	II	Rest after neoadj.th.
590	P560	-	FFPE	1	WT	38 G>A	WT	-	MSKK	65	M	C18.2	IV	Adenocarcinoma
591	P595	-	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	74	M	C18.7	III	Mucinous Carcinoma
592	P598	M28	FFPE	1	WT	35 G>C	WT	-	MSKK	45	F	C20.-	I	Adenocarcinoma
593	P606	-	FFPE	0	WT	WT	WT	-	MSKK	76	M	C20.-	III	Rest after neoadj.th.
594	P605	-	FFPE	0	WT	WT	WT	-	MSKK	53	F	C18.7	III	Adenocarcinoma
595	P581	-	FFPE	0	WT	WT	WT	-	MSKK	68	F	C20.9	II	Adenocarcinoma
596	P610	-	FFPE	1	WT	35 G>T	WT	35 G>T	MSKK	70	F	C20.-	I	Adenocarcinoma
597	P611	-	FFPE	1	1799 T>A	WT	WT	WT	MSKK	66	F	C18.6	III	Mucinous Carcinoma
598	P612	-	FFPE	2	WT	35 G>T	1633 G>A	-	MSKK	77	M	C18.7	I	Adenocarcinoma
599	P614	-	FFPE	0	WT	WT	WT	-	MSKK	73	F	C18.7	I	Adenocarcinoma
600	P615	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	72	F	C18.3	I	Mucinous Carcinoma
601	P616	M31	FFPE	2	WT	38 G>A	3140 A>G	-	MSKK	72	F	C18.7	I	Mucinous Carcinoma
602	P617	-	FFPE	0	WT	WT	WT	-	MSKK	56	M	C20.9	I	Adenocarcinoma
603	P618	-	FFPE	1	WT	35 G>C	WT	-	MSKK	71	M	C18.0	III	Adenocarcinoma

604	P619	-	FFPE	1	WT	436 G>A	WT	-	MSKK	74	M	C20.9	I	Mucinous Carcinoma
605	P620	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	58	M	C18.0	II	Mucinous Carcinoma
606	P621	M33	FFPE	1	1799 T>A	WT	WT	-	MSKK	71	M	C18.7	II	Adenocarcinoma
607	P623	-	FFPE	1	WT	WT	3140 A>G	-	MSKK	79	M	C18.7	I	Adenocarcinoma
608	P624	M34	FFPE	0	WT	WT	WT	-	MSKK	68	M	C18.7	IV	Adenocarcinoma
609	P625	-	FFPE	0	WT	WT	WT	-	MSKK	66	F	C18.3	II	Adenocarcinoma
610	P626	-	FFPE	2	1799 T>A	WT	1633 G>A	WT	MSKK	88	F	C18.2	II	Unusual Differentiation
611	P597	-	FFPE	0	WT	WT	WT	-	MSKK	87	F	C20.-	-	Tumour in Adenoma
612	P629	-	FFPE	2	WT	35 G>T	3140 A>G	-	MSKK	63	M	C20.9	II	Adenocarcinoma
613	P630	-	FFPE	0	WT	WT	WT	-	MSKK	75	M	C18.4	II	Adenocarcinoma
614	P608	-	FFPE	0	WT	WT	WT	-	MSKK	57	M	C20.-	I	Adenocarcinoma
615	P609	-	FFPE	0	WT	WT	WT	-	MSKK	75	M	C20.9	I	Adenocarcinoma
616	P632	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	87	F	C18.2	III	Adenocarcinoma
617	P633	M39	FFPE	0	WT	WT	WT	-	MSKK	87	F	C18.7	II	Adenocarcinoma
618	P634	M40	FFPE	0	WT	WT	WT	-	MSKK	68	F	C18.7	III	Adenocarcinoma
619	P636	-	FFPE	0	WT	WT	WT	-	MSKK	73	M	C18.7	III	Synchronous CRC
620	P622	-	FFPE	1	WT	WT	1633 G>A	-	MSKK	60	M	C18.0	I	Mixed Diff.with Mucin
621	P257	-	FFPE	1	WT	38 G>A	WT	-	MSKK	51	M	C18.4	III	Adenocarcinoma
622	P256	-	FFPE	2	1799 T>A	WT	3140 A>G	-	MSKK	81	F	C18.4	III	Adenocarcinoma
623	P627	M37	FFPE	1	WT	WT	1633 G>A	-	MSKK	74	M	C18.2	IV	Adenosquamous carcinoma
624	P628	M38	FFPE	0	WT	WT	WT	-	MSKK	64	M	C18.2	IV	Mixed Diff.with Mucin
625	P603	-	FFPE	1	WT	35 G>A	WT	-	MSKK	85	M	C18.4	II	Mucinous Carcinoma
626	P640	-	FFPE	1	WT	34 G>A	WT	-	MSKK	67	M	C18.0	II	Adenocarcinoma
627	P641	-	FFPE	0	WT	WT	WT	-	MSKK	71	M	C18.2	III	Adenocarcinoma
628	P604	-	FFPE	2	WT	35 G>A	1624 G>A	-	MSKK	57	M	C18.0	II	Adenocarcinoma
629	P645	-	FFPE	0	WT	WT	WT	WT	MSKK	78	M	C18.7	II	Adenocarcinoma
630	P471	-	FFPE	1	WT	35 G>T	WT	-	MSKK	74	F	C20.-	II	Adenocarcinoma
631	P607	-	FFPE	2	WT	35 G>C	1633 G>A	-	MSKK	87	M	C18.0	II	Adenocarcinoma
632	P647	-	FFPE	0	WT	WT	WT	-	MSKK	57	M	C19.9	III	Adenocarcinoma
633	P648	-	FFPE	1	WT	35 G>T	WT	-	MSKK	50	F	C18.3	II	Adenocarcinoma
634	P631	-	FFPE	1	WT	38 G>A	WT	38 G>A	MSKK	83	M	C18.5	III	Mucinous Carcinoma
635	P651	-	FFPE	1	WT	37 G>T	WT	37 G>T	MSKK	75	M	C18.0	III	Adenocarcinoma
636	P653	-	FFPE	0	WT	WT	WT	-	MSKK	59	F	C20.9	III	Neuroendocrine Diff.
637	P654	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	84	M	C18.5	III	Neuroendocrine Diff.

638	P642	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	50	M	C20.9	III	Unusual Differentiation
639	P644	-	FFPE	0	WT	WT	WT	-	MSKK	52	M	C18.4	II	Adenocarcinoma
640	P643	-	FFPE	0	WT	WT	WT	-	MSKK	84	M	C18.4	I	Adenocarcinoma
641	P639	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	66	F	C18.0	II	Adenocarcinoma
642	P600	-	FFPE	1	WT	436 G>A	WT	-	MSKK	48	M	C18.2	IV	Adenosquamous carcinoma
643	P656	-	FFPE	0	WT	WT	WT	-	MSKK	59	M	C20.-	II	Rest after neoadj.th.
644	P649	M41	FFPE	0	WT	WT	WT	-	MSKK	60	F	C18.4	III	Signet-Ring-Cell Ca.
645	P657	-	FFPE	1	WT	38 G>A	WT	-	MSKK	84	F	C18.4	IV	Adenocarcinoma
646	P658	-	FFPE	0	WT	WT	WT	-	MSKK	77	M	C19.9	II	Adenocarcinoma
647	P659	-	FFPE	1	WT	WT	1624 G>A	-	MSKK	67	M	C20.-	I	Mucinous Carcinoma
648	P652	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C18.2	II	Adenocarcinoma
649	P470	-	FFPE	2	WT	35 G>A	3140 A>G	-	MSKK	64	M	C18.6	-	Tumour in Adenoma
650	P646	-	FFPE	1	WT	34 G>A	WT	-	MSKK	75	F	C18.7	II	Adenocarcinoma
651	P660	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	71	F	C18.5	III	Adenocarcinoma
652	P662	M43	FFPE	1	1799 T>A	WT	WT	-	MSKK	78	F	C18.3	I	Mixed Diff.with Mucin
653	P638	-	FFPE	0	WT	WT	WT	-	MSKK	71	M	C20.9	I	Adenocarcinoma
654	P664	M44	FFPE	0	WT	WT	WT	-	MSKK	53	F	C19.9	I	Adenocarcinoma
655	P635	-	FFPE	0	WT	WT	WT	-	MSKK	77	F	C18.6	I	Adenocarcinoma
656	P665	-	FFPE	1	WT	WT	1624 G>A		MSKK	82	F	C18.6	II	Synchronous CRC
657	P666	-	FFPE	1	WT	35 G>A	WT	-	MSKK	82	F	C18.0	II	Adenocarcinoma
658	P670	-	FFPE	2	WT	35 G>T	1624 G>A	-	MSKK	80	M	C18.7	IV	Adenocarcinoma
659	P672	-	FFPE	0	WT	WT	WT	-	MSKK	72	F	C18.6	II	Adenocarcinoma
660	P661	-	FFPE	0	WT	WT	WT	-	MSKK	75	M	C20.9	III	Mucinous Carcinoma
661	P667	-	FFPE	0	WT	WT	WT	-	MSKK	75	F	C20.9	III	Unusual Differentiation
662	P682	-	FFPE	0	WT	WT	WT	-	MSKK	68	M	C20.-	IV	Adenocarcinoma
663	P675	-	FFPE	2	WT	34 G>A	1624 G>A	-	MSKK	74	M	C20.-	II	Mucinous Carcinoma
664	P676	-	FFPE	0	WT	WT	WT	-	MSKK	62	M	C18.6	II	Adenocarcinoma
665	P687	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	88	F	C18.2	I	Adenocarcinoma
666	P690	-	FFPE	0	WT	WT	WT	-	MSKK	87	F	C18.0	II	Adenocarcinoma
667	P691	-	FFPE	0	WT	WT	WT	-	MSKK	76	M	C20.9	III	Adenocarcinoma
668	P692	M55	FFPE	0	WT	WT	WT	-	MSKK	93	M	C18.0	III	Adenocarcinoma
669	P679	M50	FFPE	0	WT	WT	WT	-	MSKK	73	M	C18.7	III	Adenocarcinoma
670	P680	M51	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	55	F	C18.3	III	Adenocarcinoma
671	P681	-	FFPE	0	WT	WT	WT	-	MSKK	82	F	C18.7	II	Adenocarcinoma

672	P673	M49	FFPE	1	WT	35 G>C	WT	-	MSKK	77	F	C18.0	IV	Adenocarcinoma
673	P693	M56	FFPE	1	WT	35 G>T	WT	-	MSKK	55	F	C20.9	I	Adenocarcinoma
674	P694	-	FFPE	0	WT	WT	WT	WT	MSKK	60	M	C18.7	II	Adenocarcinoma
675	P688	-	FFPE	0	WT	WT	WT	NA	MSKK	82	F	C18.7	II	Adenocarcinoma
676	P689	-	FFPE	1	WT	436 G>A	WT	WT	MSKK	73	M	C18.3	IV	Adenocarcinoma
677	P700	-	FFPE	0	WT	WT	WT	-	MSKK	71	F	C18.3	III	Neuroendocrine Diff.
678	P655	-	FFPE	0	WT	WT	WT	-	MSKK	59	M	C18.7	II	Adenocarcinoma
679	P701	M58	FFPE	1	WT	WT	1633 G>A	-	MSKK	71	M	C18.7	II	Adenocarcinoma
680	P702	M59	FFPE	1	1799 T>A	WT	WT	-	MSKK	75	F	C18.3	III	Adenocarcinoma
681	P703	-	FFPE	1	WT	38 G>A	WT	-	MSKK	49	F	C18.0	III	Undifferentiated
682	P708	M61	FFPE	1	WT	35 G>T	WT	-	MSKK	70	M	C18.2	I	Adenocarcinoma
683	P710	M63	FFPE	1	WT	38 G>A	WT	-	MSKK	92	F	C18.0	III	Adenocarcinoma
684	P712	M66	FFPE	0	WT	WT	WT	-	MSKK	63	M	C18.2	II	Adenocarcinoma
685	P709	M62	FFPE	0	WT	WT	WT	-	MSKK	68	F	C20.9	III	Adenocarcinoma
686	P699	-	FFPE	1	WT	WT	1624 G>A	-	MSKK	87	F	C18.0	I	Adenocarcinoma
687	P697	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	57	F	C18.7	I	Tumour in Adenoma
688	P704	M60	FFPE	0	WT	WT	WT	-	MSKK	85	M	C20.9	III	Adenocarcinoma
689	P663	-	FFPE	1	WT	38 G>A	WT	-	MSKK	71	M	C18.2	III	Synchronous CRC
690	P716	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C18.7	I	Mucinous Carcinoma
691	P683	-	FFPE	1	WT	35 G>T	WT	35 G>T	MSKK	54	M	C18.7	III	Adenocarcinoma
692	P684	-	FFPE	0	WT	WT	WT	-	MSKK	55	M	C19.9	II	Adenocarcinoma
693	P686	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	72	F	C18.3	IV	Adenocarcinoma
694	P707	-	FFPE	2	1799 T>A	WT	3140 A>G	-	MSKK	76	M	C18.5	III	Mucinous Carcinoma
695	P705	-	FFPE	1	WT	35 G>A	WT	-	MSKK	78	F	C18.0	III	Adenocarcinoma
696	P711	M64	FFPE	1	WT	35 G>A	WT	-	MSKK	65	M	C18.0	IV	Adenocarcinoma
697	P719	-	FFPE	0	WT	WT	WT	-	MSKK	63	F	C18.7	I	Adenocarcinoma
698	P721	-	FFPE	1	WT	35 G>T	WT	-	MSKK	76	M	C20.9	III	Rest after neoadj.th.
699	P723	-	FFPE	0	WT	WT	WT	-	MSKK	84	M	C18.5	II	Adenocarcinoma
700	P724	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C18.4	III	Synchronous CRC
701	P725	-	FFPE	1	WT	35 G>T	WT	-	MSKK	66	M	C18.7	I	Adenocarcinoma
702	P726	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C20.-	II	Rest after neoadj.th.
703	P727	-	FFPE	0	WT	WT	WT	-	MSKK	72	F	C20.-	I	Adenocarcinoma
704	P714	-	FFPE	0	WT	WT	WT	-	MSKK	67	F	C20.-	II	Rest after neoadj.th.
705	P567	M16	FFPE	0	WT	WT	WT	-	MSKK	70	M	C18.7	IV	Adenocarcinoma

706	P514	-	FFPE	2	WT	35 G>A	1624 G>A	-	MSKK	73	M	C18.7	I	Adenocarcinoma
707	P729	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C18.4	II	Adenocarcinoma
708	P720	-	FFPE	1	WT	34 G>T	WT	-	MSKK	52	F	C18.7	III	Adenocarcinoma
709	P730	-	FFPE	0	WT	WT	WT	WT	MSKK	68	M	C18.7	I	Adenocarcinoma
710	P732	-	FFPE	0	WT	WT	WT	-	MSKK	73	F	C18.7	I	Adenocarcinoma
711	P733	M68	FFPE	1	WT	35 G>T	WT	35 G>T	MSKK	72	M	C20.9	III	Adenocarcinoma
712	P734	M69	FFPE	1	WT	WT	1624 G>A	-	MSKK	65	M	C18.5	II	Adenocarcinoma
713	P735	-	FFPE	1	WT	35 G>T	WT	35 G>T	MSKK	75	M	C18.7	II	Adenocarcinoma
714	P736	-	FFPE	1	1799 T>A	WT	WT	WT	MSKK	78	F	C18.0	II	Mucinous Carcinoma
715	P715	-	FFPE	1	WT	34 G>A	WT	-	MSKK	85	F	C20.9	II	Adenocarcinoma
716	P717	M67	FFPE	0	WT	WT	WT	-	MSKK	72	M	C18.7	II	Adenocarcinoma
717	P698	M57	FFPE	1	WT	38 G>A	WT	-	MSKK	87	F	C20.-	III	Adenocarcinoma
718	P737	M71	FFPE	0	WT	WT	WT	-	MSKK	62	M	C18.7	III	Adenocarcinoma
719	P713	-	FFPE	0	WT	WT	WT	-	MSKK	63	M	C20.-	I	Rest after neoadj.th.
720	P741	-	FFPE	1	WT	WT	1624 G>A	-	MSKK	66	M	C18.7	IV	Adenocarcinoma
721	P742	-	FFPE	2	WT	436 G>A	3140 A>G	-	MSKK	68	M	C18.0	I	Adenocarcinoma
722	P745	-	FFPE	0	WT	WT	WT	-	MSKK	79	F	C20.-	I	Adenocarcinoma
723	P746	M73	FFPE	0	WT	WT	WT	-	MSKK	73	F	C18.7	II	Adenocarcinoma
724	P747	-	FFPE	0	WT	WT	WT	-	MSKK	64	M	C18.2	IV	Adenocarcinoma
725	P748	-	FFPE	1	WT	34 G>T	WT	-	MSKK	51	F	C18.0	III	Adenocarcinoma
726	P718	-	FFPE	0	WT	WT	WT	-	MSKK	53	F	C20.9	III	Rest after neoadj.th.
727	P706	-	FFPE	0	WT	WT	WT	-	MSKK	55	F	C18.0	III	Adenocarcinoma
728	P728	-	FFPE	0	WT	WT	WT	-	MSKK	54	M	C18.7	III	Mixed Diff.with Mucin
729	P750	-	FFPE	0	WT	WT	WT	-	MSKK	73	M	C19.9	I	Adenocarcinoma
730	P751	-	FFPE	1	WT	35 G>T	WT	-	MSKK	72	M	C18.0	II	Adenocarcinoma
731	P722	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	68	F	C21.8	I	Adenocarcinoma
732	P753	-	FFPE	0	WT	WT	WT	-	MSKK	69	M	C20.9	I	Rest after neoadj.th.
733	P754	-	FFPE	1	WT	35 G>A	WT	-	MSKK	73	F	C18.0	I	Mixed Diff.with Mucin
734	P738	-	FFPE	1	WT	38 G>A	WT	-	MSKK	64	M	C20.-	II	Adenocarcinoma
735	P740	-	FFPE	1	WT	34 G>A	WT	-	MSKK	70	M	C18.0	II	Mucinous Carcinoma
736	P739	-	FFPE	0	WT	WT	WT	-	MSKK	85	M	C20.-	II	Adenocarcinoma
737	P755	-	FFPE	1	WT	35 G>A	WT	-	MSKK	88	F	C18.7	III	Mixed Diff.with Mucin
738	P650	-	FFPE	0	WT	WT	WT	-	MSKK	78	F	C20.-	I	Adenocarcinoma
739	P671	-	FFPE	2	1799 T>A	WT	3140 A>G	-	MSKK	63	F	C18.2	I	Medullary Differentitation

740	P669	-	FFPE	0	WT	WT	WT	-	MSKK	61	M	C20.-	I	Adenocarcinoma
741	P678	-	FFPE	1	WT	38 G>A	WT	38 G>A	MSKK	64	F	C18.6	II	Mucinous Carcinoma
742	P749	-	FFPE	0	WT	WT	WT	-	MSKK	58	M	C18.4	I	Adenocarcinoma
743	P743	-	FFPE	2	WT	35 G>T	1624 G>A	-	MSKK	75	M	C18.0	III	Adenocarcinoma
744	P757	-	FFPE	1	WT	35 G>T	WT	-	MSKK	76	M	C18.2	III	Mucinous Carcinoma
745	P758	-	FFPE	0	WT	WT	WT	NA	MSKK	68	M	C20.-	II	Adenocarcinoma
746	P762	-	FFPE	1	WT	35 G>T	WT	-	MSKK	70	M	C20.-	III	Adenocarcinoma
747	P685	-	FFPE	0	WT	WT	WT	-	MSKK	75	F	C18.7	II	Adenocarcinoma
748	P731	-	FFPE	1	WT	34 G>T; 35 G>A	WT	-	MSKK	82	F	C18.7	III	Mixed Diff.with Mucin
749	P744	-	FFPE	0	WT	WT	WT	-	MSKK	51	F	C18.0	III	Mixed Differentiation
750	P763	M74	FFPE	0	WT	WT	WT	-	MSKK	43	M	C18.7	IV	Adenocarcinoma
751	P637	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	65	F	C18.0	III	Adenocarcinoma
752	P674	-	FFPE	0	WT	WT	WT	-	MSKK	67	F	C20.9	III	Mucinous Carcinoma
753	P756	-	FFPE	1	WT	35 G>A	WT	-	MSKK	60	M	C18.4	II	Adenocarcinoma
754	P769	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C18.2	III	Adenocarcinoma
755	P770	-	FFPE	1	WT	35 G>C	WT	-	MSKK	68	F	C18.6	I	Adenocarcinoma
756	P771	-	FFPE	0	WT	WT	WT	-	MSKK	80	M	C18.7	III	Adenocarcinoma
757	P772	-	FFPE	0	WT	WT	WT	-	MSKK	52	M	C20.9	I	Rest after neoadj.th.
758	P773	-	FFPE	0	WT	WT	WT	-	MSKK	54	F	C18.2	I	Tumour in Adenoma
759	P775	M75	FFPE	1	WT	35 G>C	WT	-	MSKK	79	F	C18.2	III	Adenocarcinoma
760	P776	-	FFPE	0	WT	WT	WT	-	MSKK	54	M	C18.7	III	Adenocarcinoma
761	P777	-	FFPE	2	WT	34 G>T	1633 G>A	-	MSKK	77	F	C18.0	II	Adenocarcinoma
762	P696	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	67	F	C18.2	II	Undifferentiated
763	P779	-	FFPE	1	WT	35 G>C	WT	-	MSKK	78	F	C18.6	III	Adenocarcinoma
764	P677	-	FFPE	0	WT	WT	WT	-	MSKK	75	F	C18.5	II	Adenocarcinoma
765	P695	-	FFPE	0	WT	WT	WT	-	MSKK	73	M	C18.6	II	Adenocarcinoma
766	P780	-	FFPE	2	WT	35 G>C	1633 G>A	-	MSKK	74	F	C20.9	III	Adenocarcinoma
767	P781	-	FFPE	1	1799 T>A	WT	WT	WT	MSKK	66	M	C18.3	IV	Mucinous Carcinoma
768	P668	-	FFPE	0	WT	WT	WT	-	MSKK	55	M	C20.9	III	Rest after neoadj.th.
769	P760	-	FFPE	2	WT	35 G>T	1633 G>A	35 G>T	MSKK	70	M	C18.6	II	Adenocarcinoma
770	P761	-	FFPE	0	WT	WT	WT	-	MSKK	87	M	C18.7	III	Adenocarcinoma
771	P765	-	FFPE	0	WT	WT	WT	-	MSKK	74	F	C18.6	II	Adenocarcinoma
772	P782	-	FFPE	0	WT	WT	WT	-	MSKK	78	F	C18.4	II	Adenocarcinoma
773	P764	-	FFPE	1	WT	38 G>A	WT	-	MSKK	65	F	C18.2	III	Adenocarcinoma

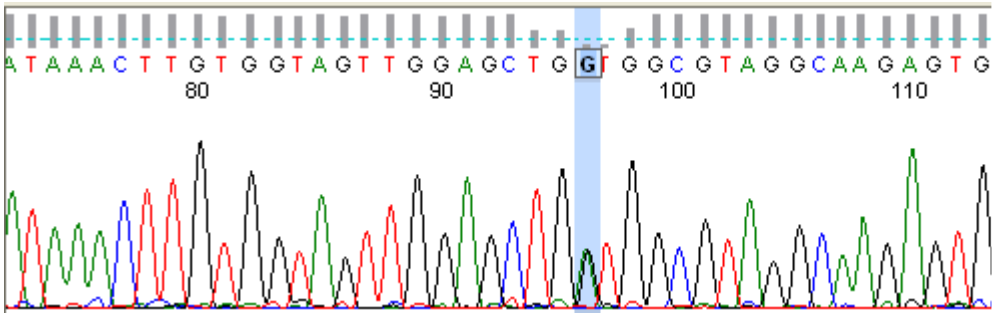
774	P783	M78	FFPE	0	WT	WT	WT	WT	MSKK	89	F	C18.7	II	Adenocarcinoma
775	P784	M79	FFPE	0	WT	WT	WT	-	MSKK	77	F	C18.2	IV	Adenocarcinoma
776	P785	-	FFPE	0	WT	WT	WT	-	MSKK	83	M	C18.7	I	Tumour in Adenoma
777	P767	-	FFPE	1	1799 T>A	WT	WT	-	MSKK	68	F	C18.4	III	Mixed Diff.with Mucin
778	P768	-	FFPE	1	WT	38 G>A	WT	-	MSKK	58	M	C20.9	IV	Adenocarcinoma
779	P786	-	FFPE	2	WT	34 G>A	1633 G>A	-	MSKK	74	M	C18.2	III	Adenocarcinoma
780	P778	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C18.6	IV	Adenocarcinoma
781	P789	-	FFPE	1	WT	35 G>A	WT	NA	MSKK	70	F	C20.9	III	Synchronous CRC
782	P790	M81	FFPE	1	1799 T>A	WT	WT	NA	MSKK	74	M	C18.7	III	Adenocarcinoma
783	P791	-	FFPE	0	WT	WT	WT	-	MSKK	59	M	C19.9	II	Adenocarcinoma
784	P787	-	FFPE	2	1799 T>A	WT	1633 G>A	-	MSKK	70	F	C18.7	III	Adenocarcinoma
785	P788	-	FFPE	2	WT	436 G>A	1633 G>A	-	MSKK	78	F	C18.0	III	Mixed Diff.with Mucin
786	P774	-	FFPE	0	WT	WT	WT	-	MSKK	40	M	C18.4	IV	Adenocarcinoma
787	P795	-	FFPE	2	WT	35 G>A	1633 G>A	-	MSKK	79	M	C20.9	II	Mucinous Carcinoma
788	P794	-	FFPE	0	WT	WT	WT	WT	MSKK	53	F	C20.9	III	Adenocarcinoma
789	P796	-	FFPE	2	WT	35 G>T	1633 G>A	-	MSKK	57	M	C19.-	I	Tumour in Adenoma
790	P797	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C18.2	III	Adenocarcinoma
791	P792	M82	FFPE	0	WT	WT	WT	-	MSKK	76	M	C18.6	I	Synchronous CRC
792	P752	-	FFPE	1	WT	35 G>A	WT	-	MSKK	61	F	C18.0	IV	Adenocarcinoma
793	P766	-	FFPE	1	WT	38 G>A	WT	-	MSKK	70	M	C20.-	I	Rest after neoadj.th.
794	P793	-	FFPE	1	WT	38 G>A	WT	-	MSKK	86	F	C18.7	II	Adenocarcinoma
795	P800	-	FFPE	1	WT	35 G>T	WT	-	MSKK	68	M	C18.7	III	Adenocarcinoma
796	P801	M83	FFPE	1	WT	35 G>C	WT	-	MSKK	68	M	C18.7	III	Adenocarcinoma
797	P804	-	FFPE	0	WT	WT	WT	-	MSKK	67	M	C18.2	II	Adenocarcinoma
798	P798	-	FFPE	0	WT	WT	WT	-	MSKK	70	M	C18.7	IV	Adenocarcinoma
799	P799	-	FFPE	0	WT	WT	WT	-	MSKK	74	M	C19.9	II	Adenocarcinoma
800	P803	-	FFPE	2	WT	35 G>T	1633 G>A	-	MSKK	74	F	C18.3	IV	Neuroendocrine Diff.
801	P809	-	FFPE	0	WT	WT	WT	-	MSKK	52	M	C20.-	II	Rest after neoadj.th.
802	P802	-	FFPE	2	WT	35 G>T	1633 G>A	-	MSKK	72	F	C18.2	III	Mucinous Carcinoma
803	P806	-	FFPE	0	WT	WT	WT	-	MSKK	66	M	C19.-	III	Adenocarcinoma
804	P808	M87	FFPE	0	WT	WT	WT	WT	MSKK	46	F	C18.6	IV	Adenocarcinoma
805	P811	-	FFPE	1	WT	35 G>C	WT	-	MSKK	65	M	C18.7	III	Adenocarcinoma
806	P812	M88	FFPE	0	WT	WT	WT	WT	MSKK	69	M	C18.4	II	Adenocarcinoma
807	P807	-	FFPE	0	WT	WT	WT	-	MSKK	71	M	C18.3	III	Adenocarcinoma

808	P810	-	FFPE	0	WT	WT	WT	-	MSKK	73	M	C19.-	III	Adenocarcinoma
809	P613	-	FFPE	1	WT	38 G>A	WT	-	MSKK	66	F	C18.2	I	Adenocarcinoma
810	P759	-	FFPE	1	WT	35 G>T	WT	-	MSKK	60	F	C20.-	II	Adenocarcinoma
811	P813	-	FFPE	0	WT	WT	WT	-	MSKK	77	M	C20.-	II	Rest after neoadj.th.
812	P814	-	FFPE	1	WT	38 G>A	WT	38 G>A	MSKK	77	F	C19.9	III	Mixed Diff.with Mucin
813	P805	-	FFPE	0	WT	WT	WT	-	MSKK	72	M	C20.-	III	Rest after neoadj.th.
814	P815	-	FFPE	1	WT	34 G>T	WT	34 G>T	MSKK	70	M	C18.6	II	Adenocarcinoma
815	P816	-	FFPE	1	WT	35 G>A	WT	35 G>A	MSKK	72	F	C20.9	III	Adenocarcinoma
816	P817	-	FFPE	0	WT	WT	WT	WT	MSKK	48	M	C20.9	III	Rest after neoadj.th.
817	P818	-	FFPE	1	WT	35 G>C	WT	-	MSKK	77	M	C18.7	I	Adenocarcinoma
818	P819	-	FFPE	0	WT	WT	WT	WT	MSKK	76	M	C18.7	III	Mixed Differentiation
819	P820	-	FFPE	1	WT	38 G>A	WT	-	MSKK	80	F	C18.7	II	Adenocarcinoma
820	P821	M93	FFPE	0	WT	WT	WT	-	MSKK	55	F	C18.7	III	Adenocarcinoma
821	P822	-	FFPE	0	WT	WT	WT	-	MSKK	76	M	C20.-	I	Adenocarcinoma
822	P823	-	Snap-frozen	1	1799 T>A	WT	WT	-	RVS	81	F	C18.0	II	Adenocarcinoma
823	P824	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	RVS	70	M	C18.0	III	Adenocarcinoma
824	P825	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	65	M	C18.0	III	Mixed Diff.with Mucin
825	P826	-	Snap-frozen	1	WT	38 G>A	WT	-	RVS	54	M	C18.4	I	Adenocarcinoma
826	P827	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	74	F	C20.9	III	Mucinous Carcinoma
827	P828	-	Snap-frozen	0	WT	WT	WT	WT	RVS	64	M	C20.9	III	Adenocarcinoma
828	P829	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	72	F	C19.9	II	Adenocarcinoma
829	P830	-	Snap-frozen	0	WT	WT	WT	WT	RVS	69	M	C20.9	III	Adenocarcinoma
830	P831	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	66	F	C18.7	III	Adenocarcinoma
831	P832	-	Snap-frozen	1	WT	35 G>C	WT	35 G>C	RVS	79	F	C19.9	III	Adenocarcinoma
832	P833	-	Snap-frozen	2	WT	35 G>T	1633 G>A	35 G>T	RVS	64	F	C18.7	II	Adenocarcinoma
833	P834	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	RVS	69	M	C18.7	III	Adenocarcinoma
834	P835	-	Snap-frozen	0	WT	WT	WT	WT	RVS	70	M	C18.7	II	Adenocarcinoma
835	P836	-	Snap-frozen	0	WT	WT	WT	WT	RVS	69	M	C19.9	II	Adenocarcinoma
836	P837	-	Snap-frozen	0	WT	WT	WT	WT	RVS	59	M	C18.7	III	Adenocarcinoma
837	P838	-	Snap-frozen	2	1799 T>A	WT	1633 G>A	WT	RVS	86	F	C18.6	III	Adenocarcinoma
838	P839	-	Snap-frozen	0	WT	WT	WT	WT	RVS	50	F	C20.9	III	Adenocarcinoma
839	P840	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	36	F	C18.7	II	Adenocarcinoma
840	P841	-	Snap-frozen	2	WT	38 G>A	1633 G>A	38 G>A	RVS	73	F	C18.2	I	Adenocarcinoma
841	P842	-	Snap-frozen	1	WT	38 G>A	WT	38 G>A	RVS	63	F	C19.-	III	Adenocarcinoma

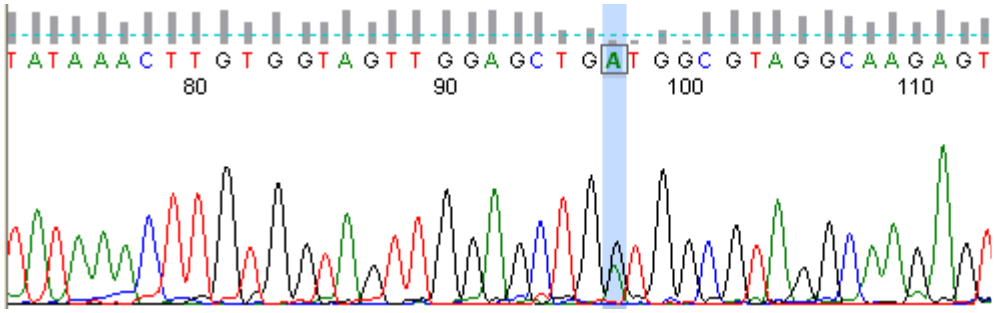
842	P843	-	Snap-frozen	2	WT	38 G>A	1633 G>A	38 G>A	RVS	70	M	C20.-	III	Adenocarcinoma
843	P844	-	Snap-frozen	1	WT	34 G>A	WT	34 G>A	RVS	62	F	C19.-	III	Adenocarcinoma
844	P845	-	Snap-frozen	2	WT	35 G>A	3140 A>G	35 G>A	RVS	79	M	C18.7	II	Adenocarcinoma
845	P846	-	Snap-frozen	1	WT	WT	1633 G>A	WT	RVS	33	F	C20.9	III	Adenocarcinoma
846	P847	-	Snap-frozen	1	WT	34 G>T	WT	34 G>T	RVS	62	M	C18.7	II	Adenocarcinoma
847	P848	-	Snap-frozen	1	WT	34 G>A	WT	34 G>A	RVS	55	M	C20.9	II	Adenocarcinoma
848	P849	-	Snap-frozen	0	WT	WT	WT	WT	RVS	67	M	C18.4	III	Signet-Ring-Cell Ca.
849	P850	-	Snap-frozen	0	WT	WT	WT	-	RVS	42	M	C20.9	I	Adenocarcinoma
850	P851	-	Snap-frozen	1	WT	34 G>A	WT	-	RVS	76	F	C20.-	III	Adenocarcinoma
851	P852	-	Snap-frozen	2	1799 T>A	WT	1624 G>A	WT	RVS	80	F	C18.0	II	Adenocarcinoma
852	P853	-	Snap-frozen	0	WT	WT	WT	WT	RVS	76	F	C20.9	II	Adenocarcinoma
853	P854	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	67	F	C18.7	IV	Adenocarcinoma
854	P855	-	Snap-frozen	2	WT	35 G>A	1633 G>A	35 G>A	RVS	65	M	C18.0	II	Adenocarcinoma
855	P856	-	Snap-frozen	1	WT	35 G>A	WT	35 G>A	RVS	53	M	C18.2	IV	Adenocarcinoma
856	P857	-	Snap-frozen	1	WT	34 G>A	WT	34 G>A	RVS	62	M	C20.9	III	Adenocarcinoma
857	P858	-	Snap-frozen	1	WT	34 G>A	WT	34 G>A	RVS	71	F	C18.7	IV	Adenocarcinoma
858	P859	-	Snap-frozen	0	WT	WT	WT	WT	RVS	58	M	C18.3	II	Adenocarcinoma
859	P860	-	Snap-frozen	1	1799 T>A	WT	WT	WT	RVS	57	M	C18.2	III	Adenocarcinoma
860	P861	-	Snap-frozen	0	WT	WT	WT	WT	RVS	71	M	C20.9	IV	Adenocarcinoma
861	P862	-	Snap-frozen	0	WT	WT	WT	WT	RVS	62	F	C18.7; C18.3	IV	Adenocarcinoma
862	P863	-	Snap-frozen	1	WT	35 G>T	WT	35 G>T	RVS	64	M	C18.7	IV	Adenocarcinoma
863	P864	-	Snap-frozen	1	1799 T>A	WT	WT	WT	RVS	68	F	C18.7	IV	Adenocarcinoma
864	P4	-	Snap-frozen	2	WT	35 G>A	3140 A>G	35 G>A	MSKK	55	F	C18.7	III	Adenocarcinoma

Tab.S6. *Sequencing chromatograms of KRAS codon 12 and 13 of the samples with matched real-time PCR results*

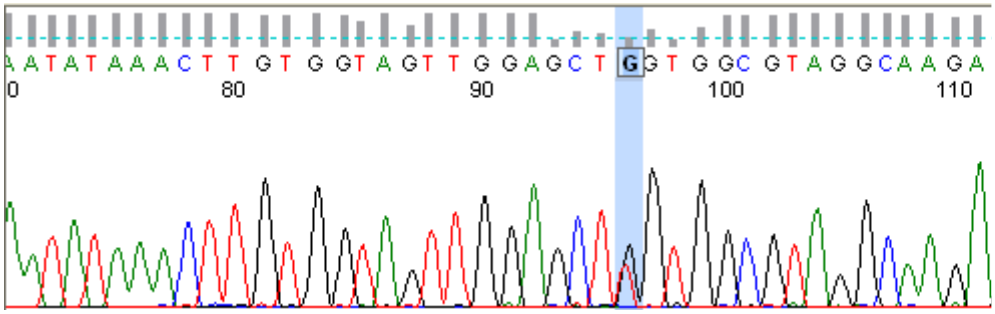
P4 35 G>A



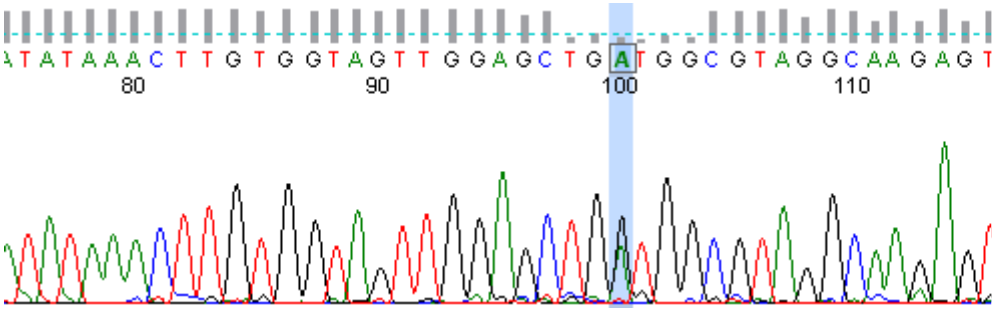
P6 35 G>A



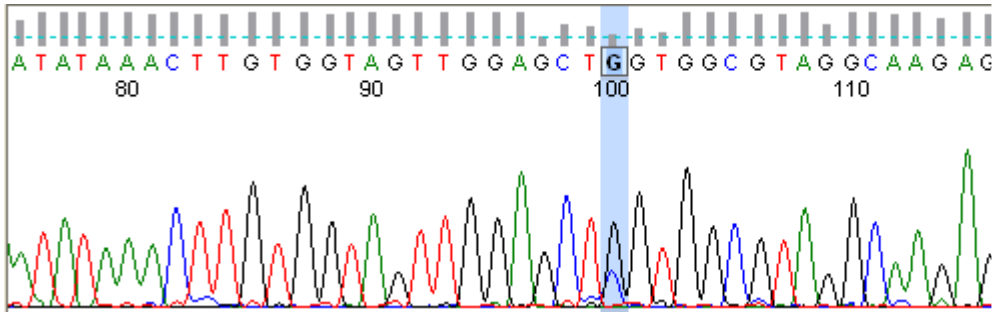
P15 34 G>T



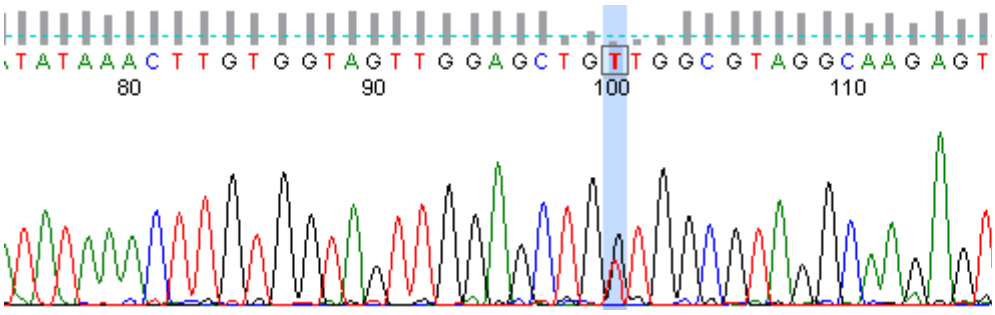
P16 35 G>A



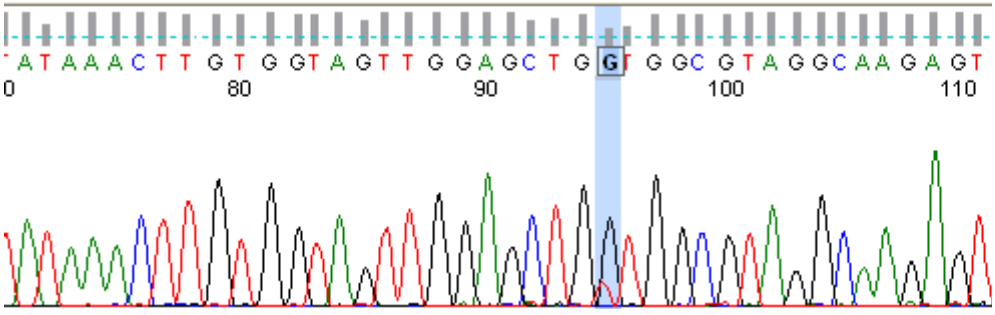
P37 34 G>C



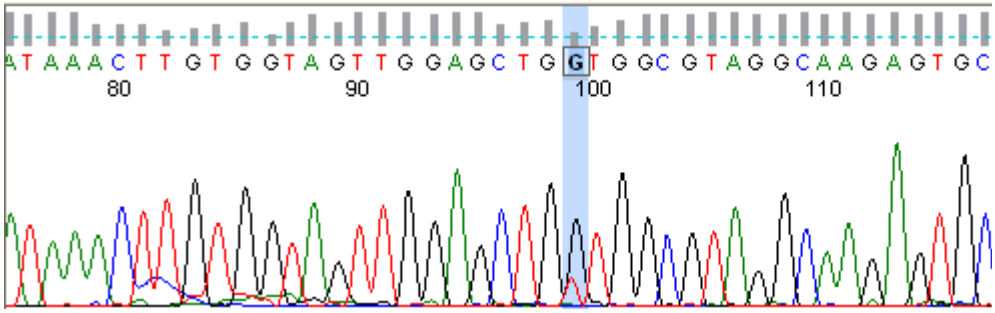
P39 35 G>T



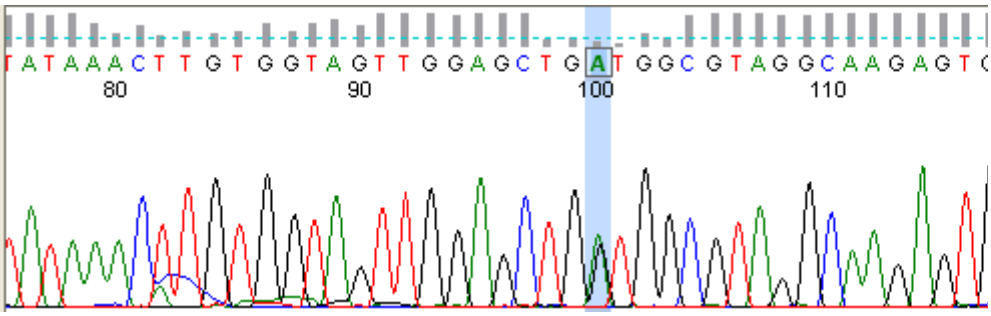
P41 35 G>T



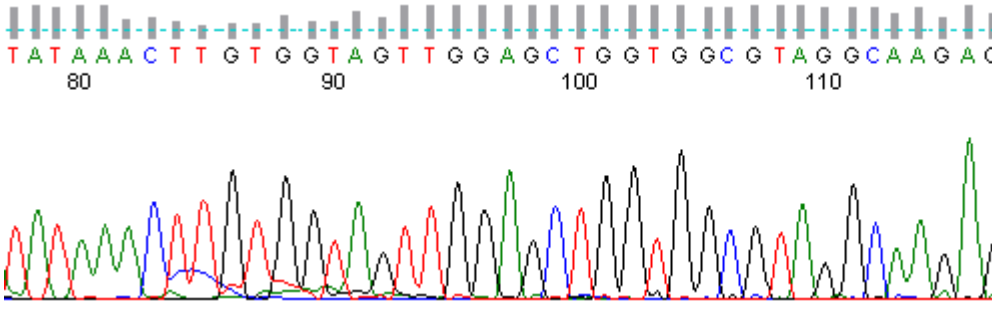
P42 35 G>T



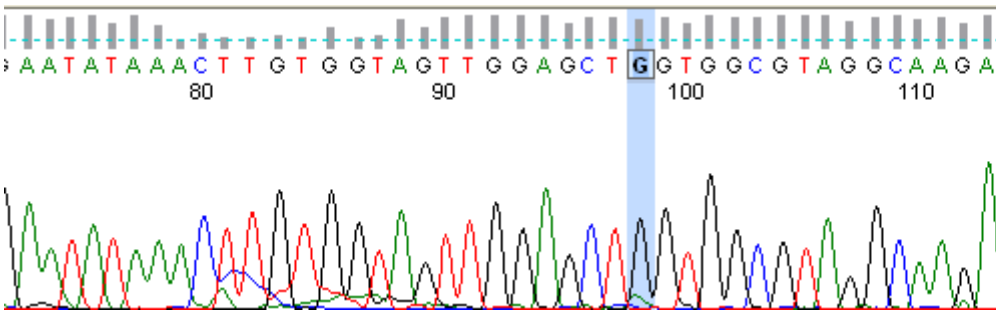
P48 35 G>A



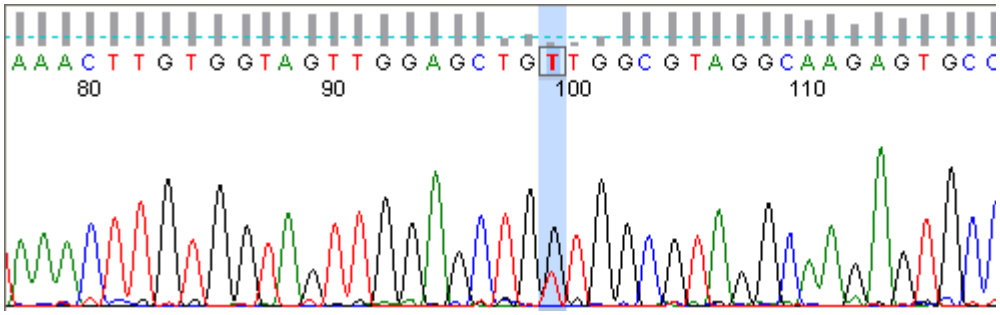
P59 WT



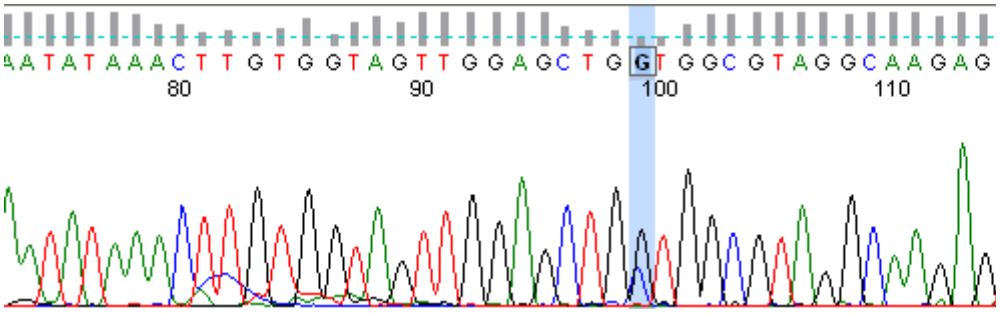
P60 unclear; allele-specific PCR: 34 G>A



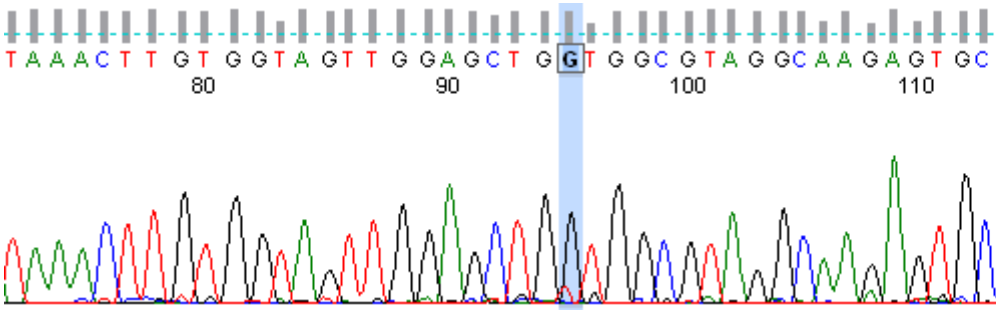
P63 35 G>T



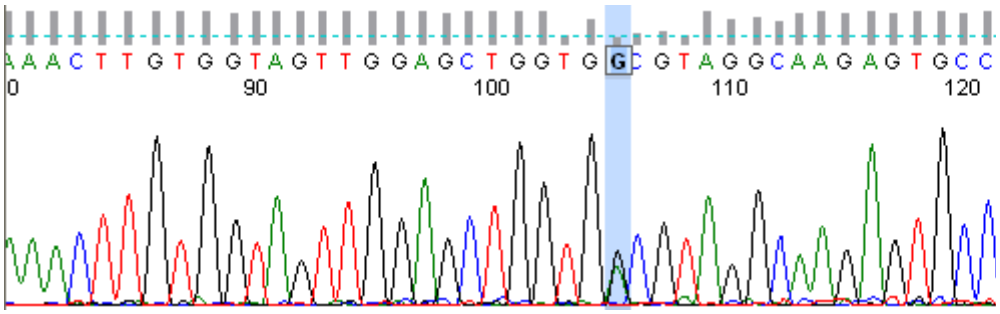
P66 35 G>C



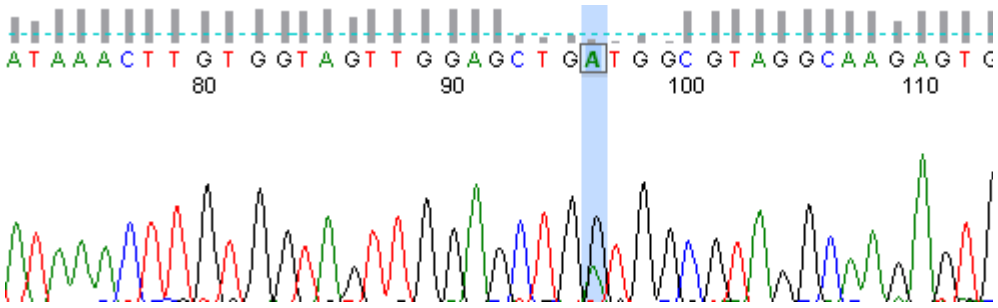
P9 unclear; allele-specific PCR: 35 G>T



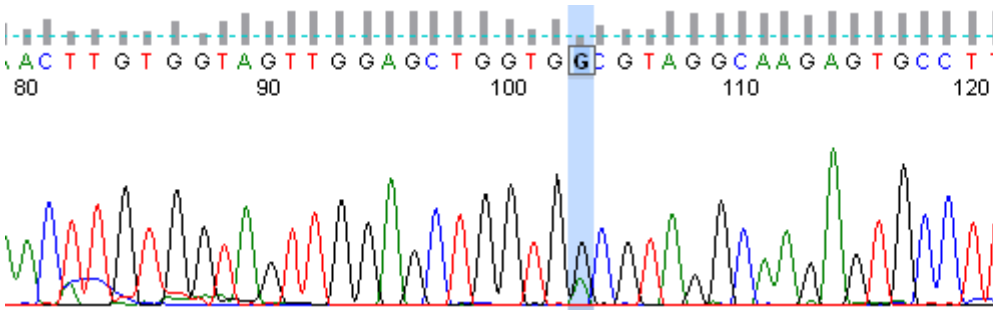
P24 38 G>A



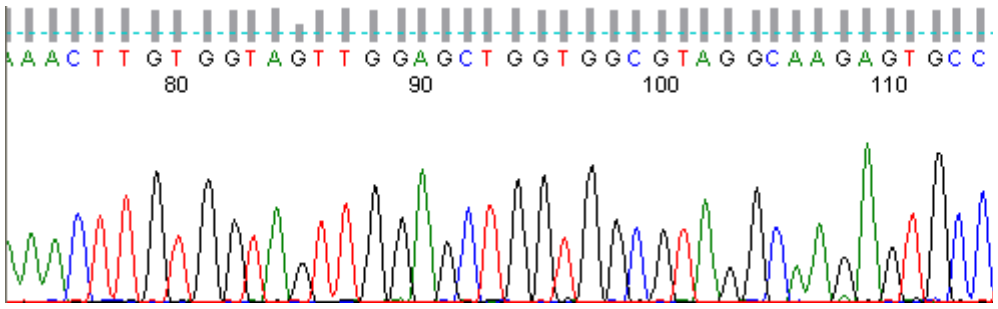
P70 35 G>A



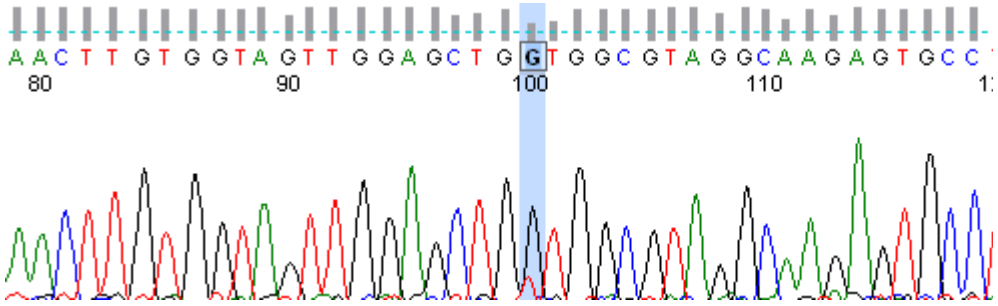
P72 38 G>A



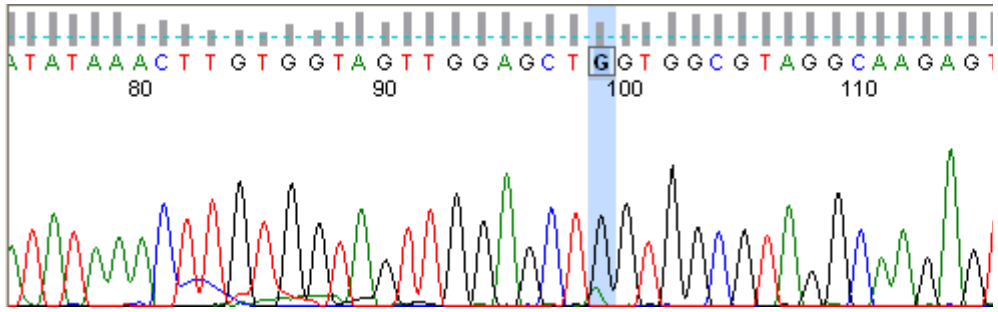
P78 WT



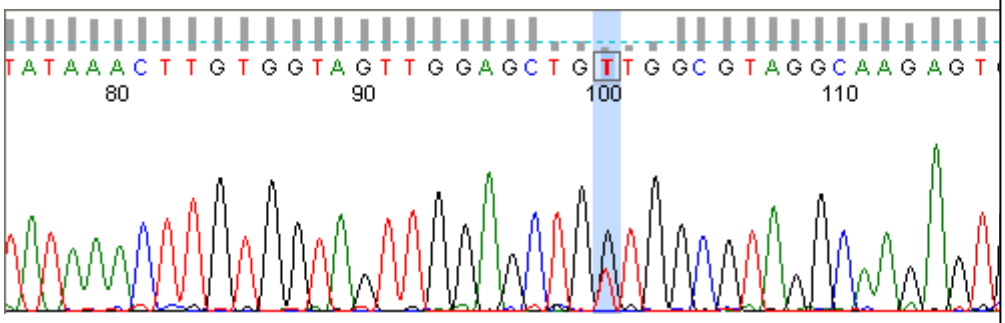
P81 35 G>T



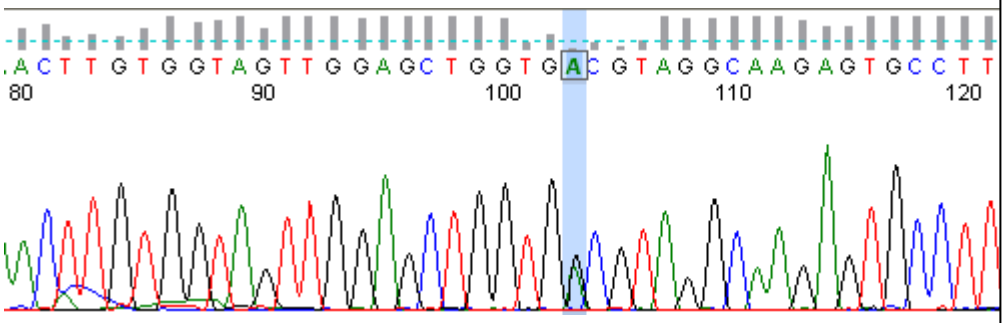
P85 34 G>A



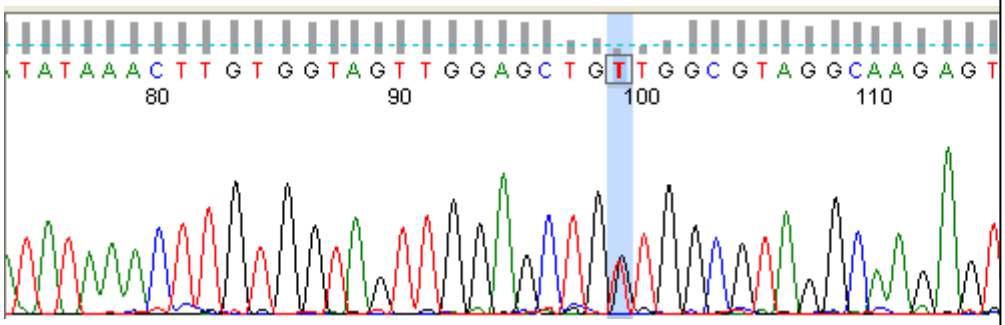
P87 35 G>T



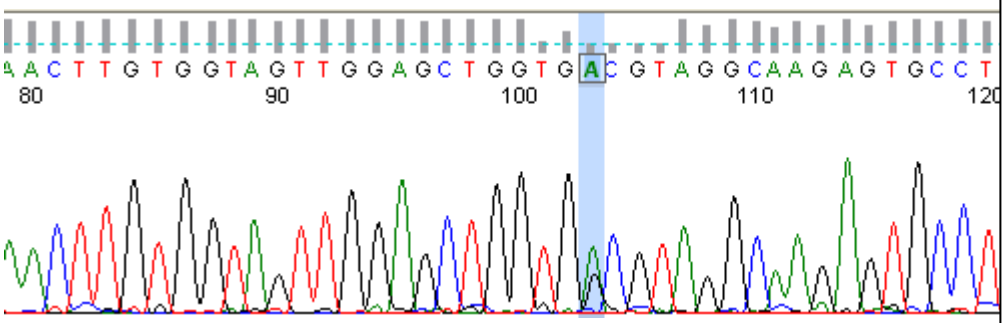
P89 38 G>A



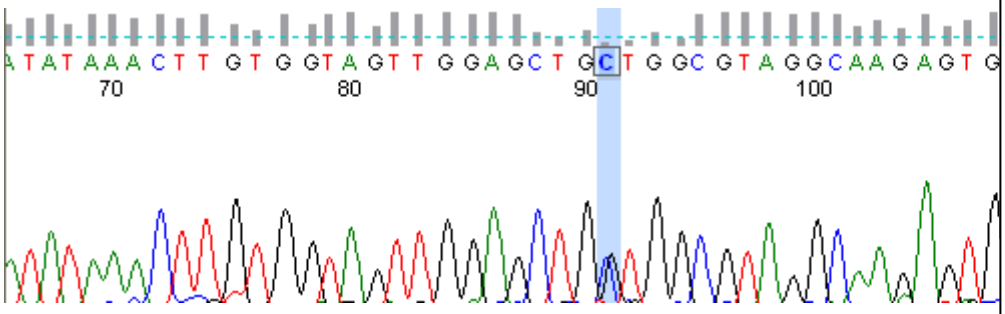
P92 35 G>T



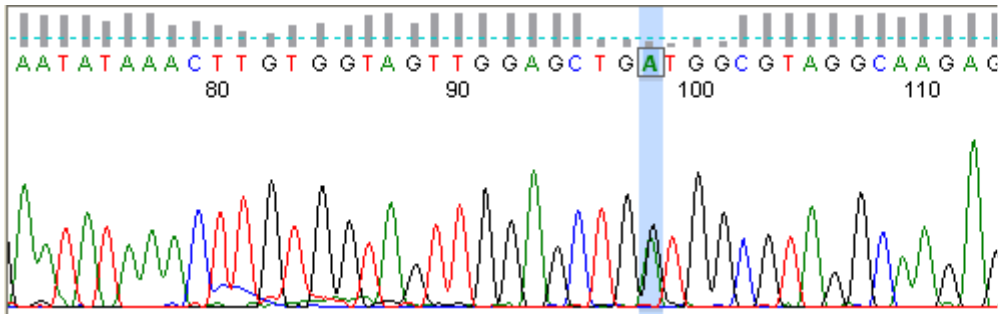
P93 38 G>A



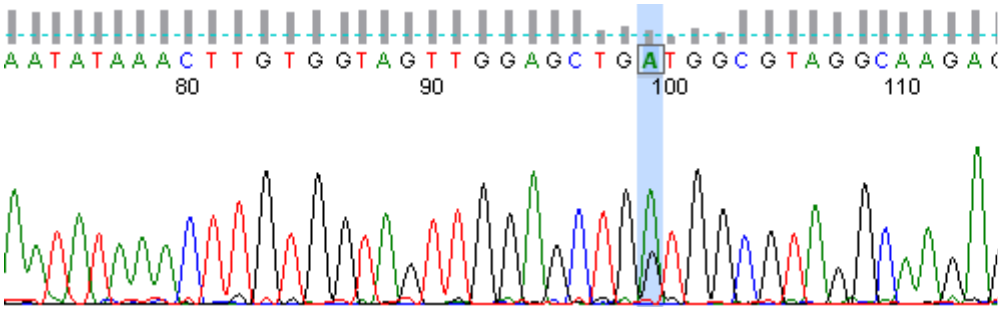
P95 35 G>C



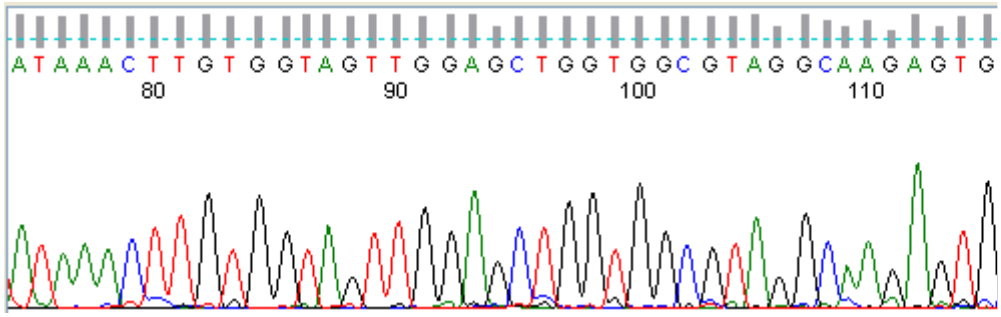
P97 35 G>A



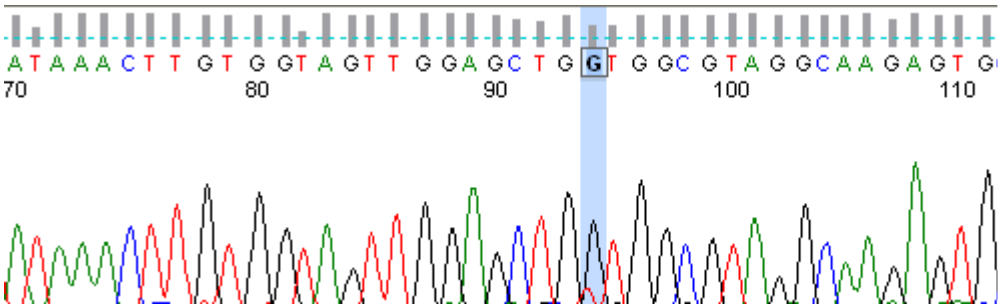
P102 35 G>A



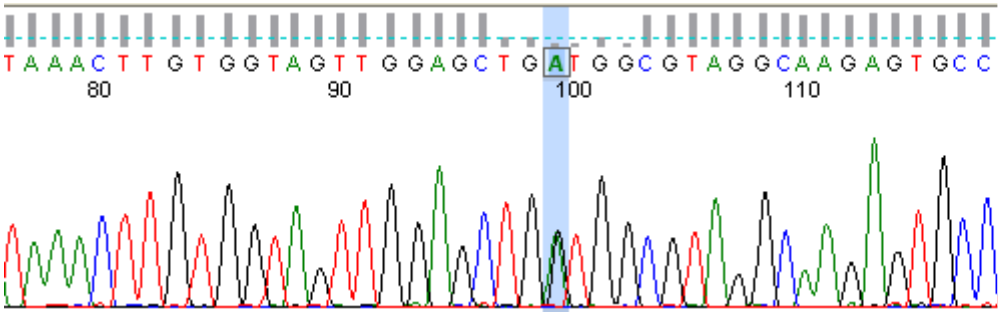
P103 WT



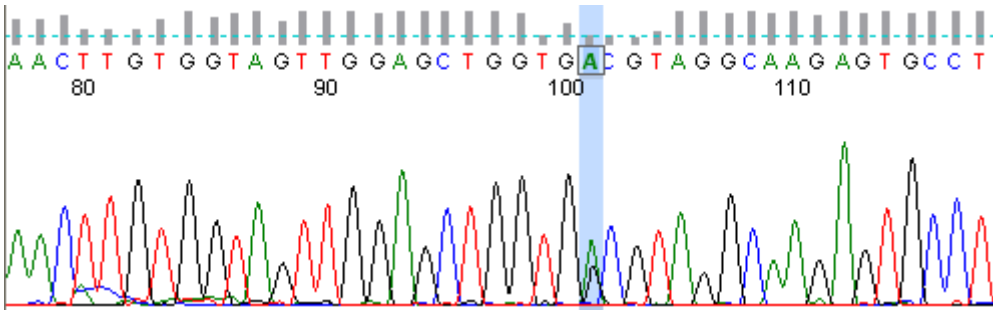
P104 35 G>T



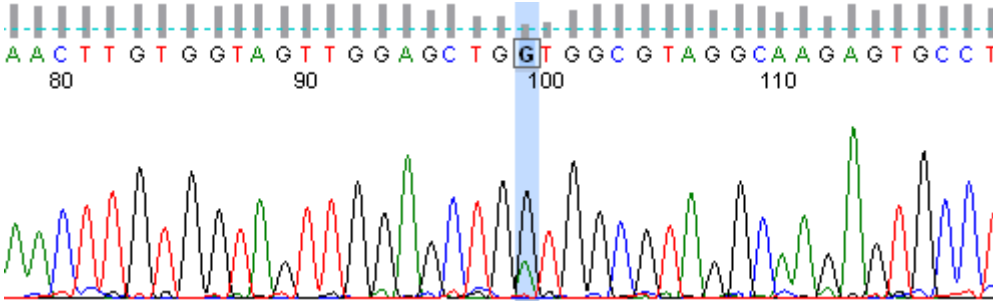
P105 35 G>A



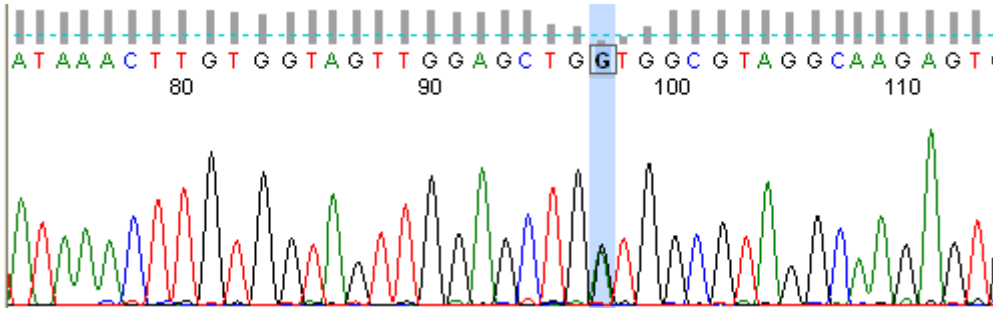
P114 38 G>A



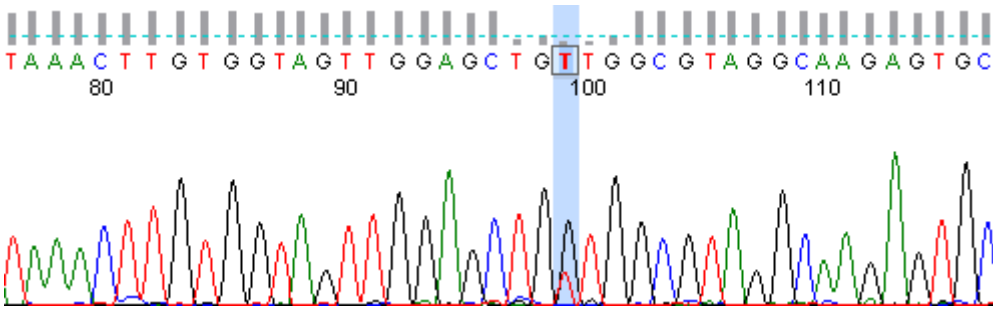
P120 35 G>A



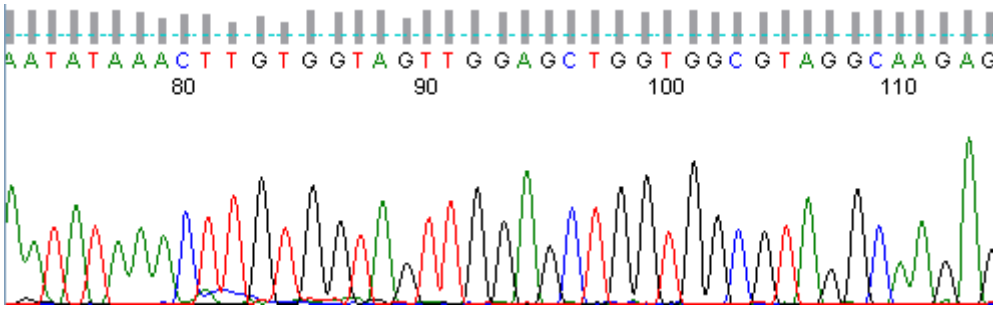
P124 35 G>A



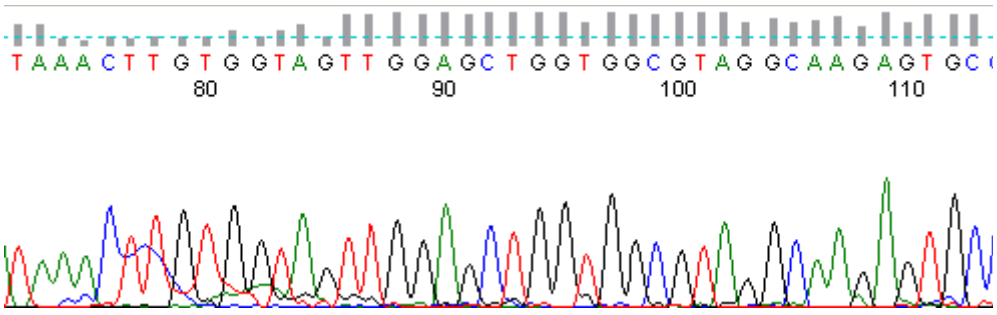
P127 35 G>T



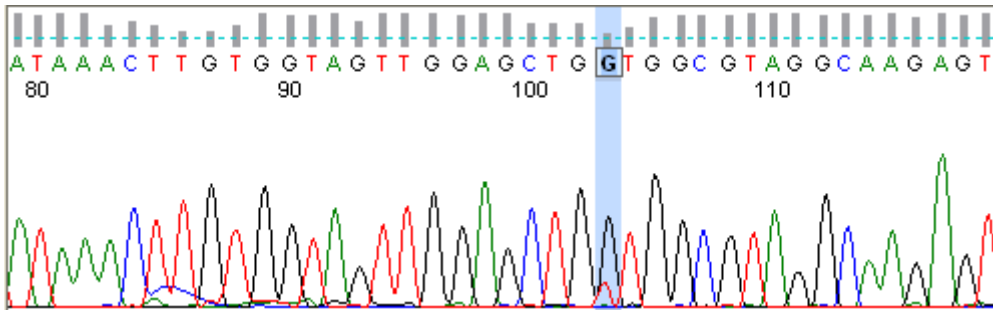
P131 WT



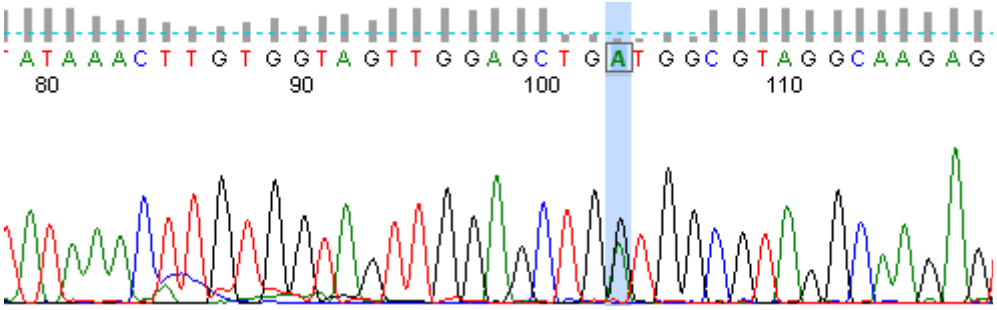
P132 WT



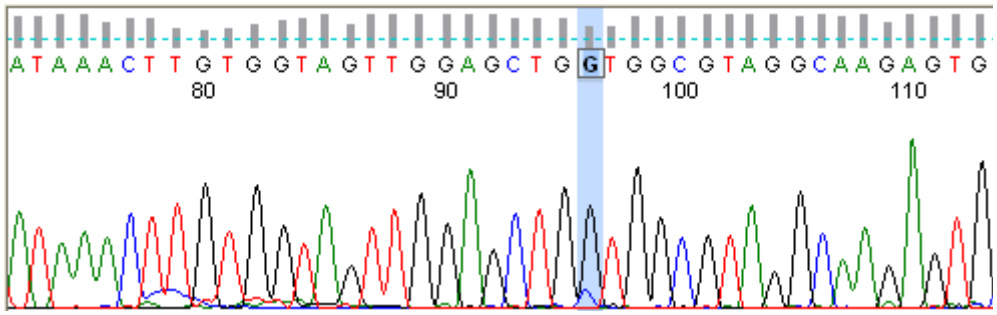
P133 35 G>T



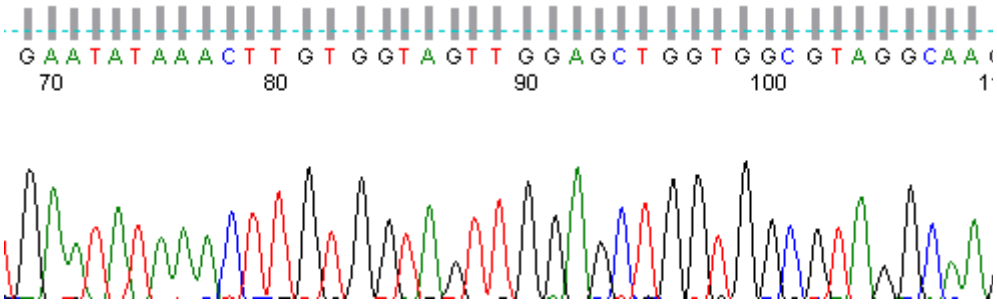
P134 35 G>A



P135 35 G>C



P137 WT



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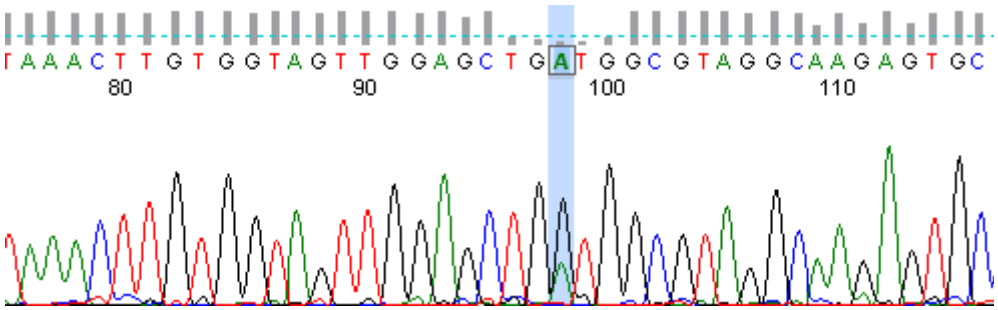
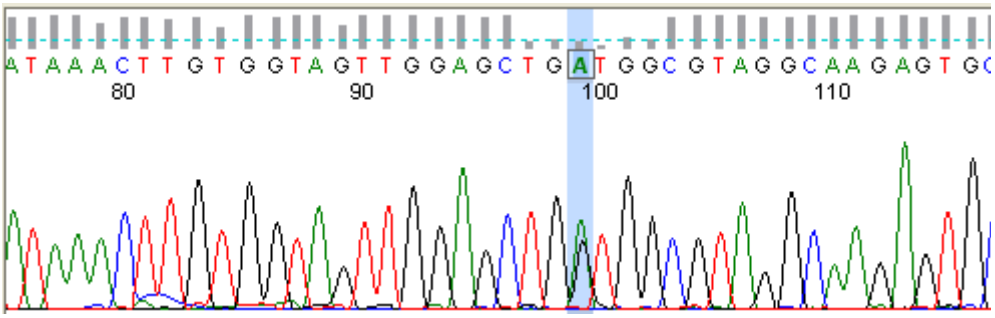
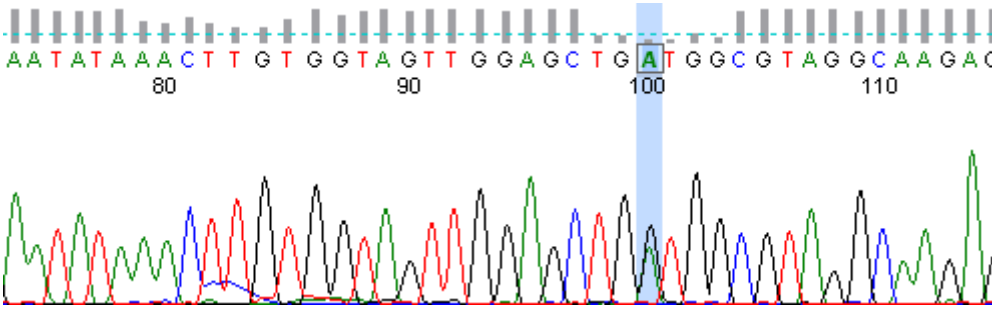


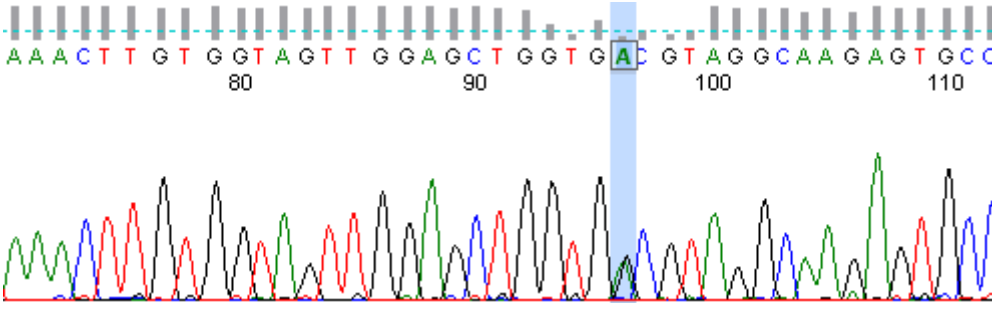
P160 35 G>A



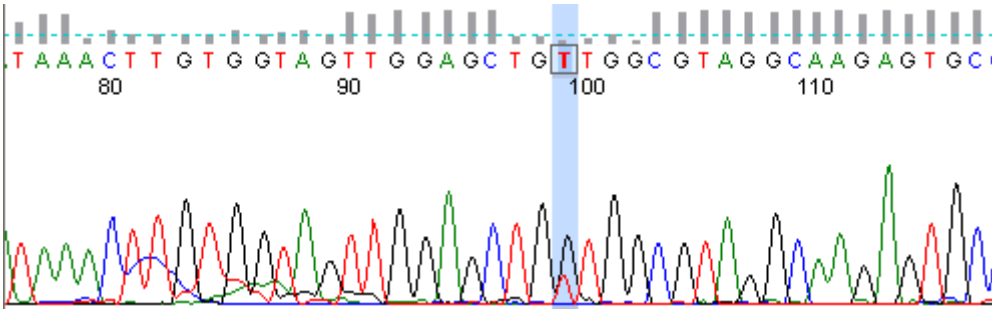
P162 35 G>A



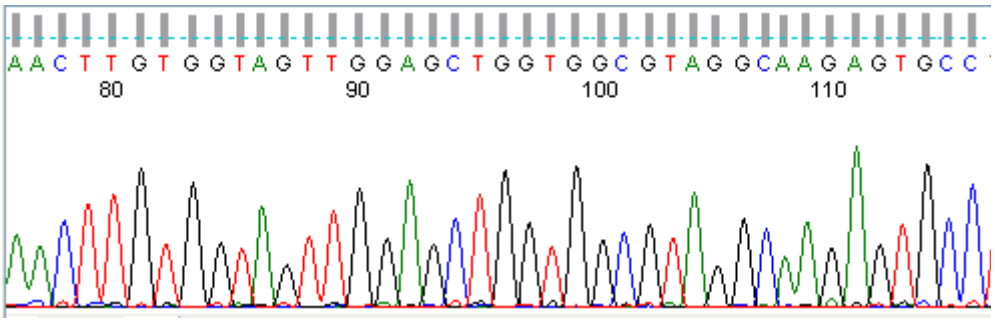
P168 38 G>A



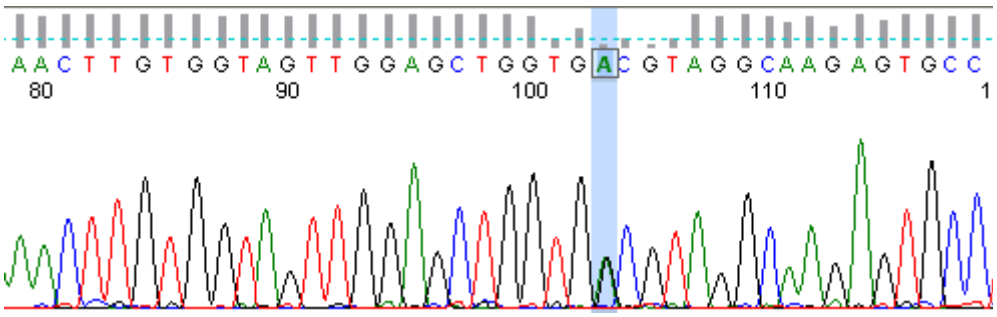
P169 35 G>T



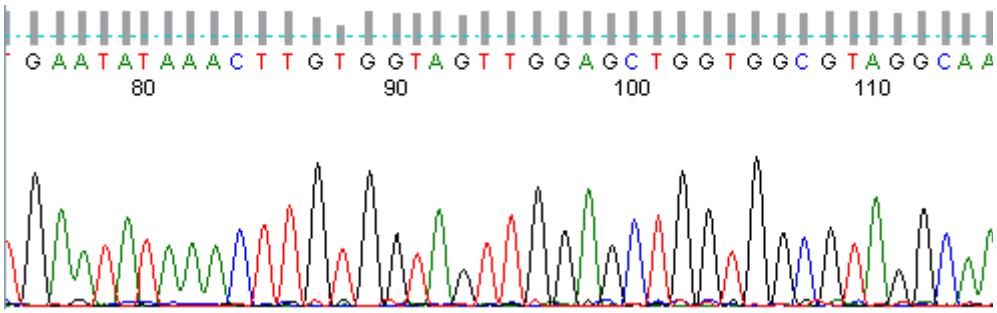
P172 WT



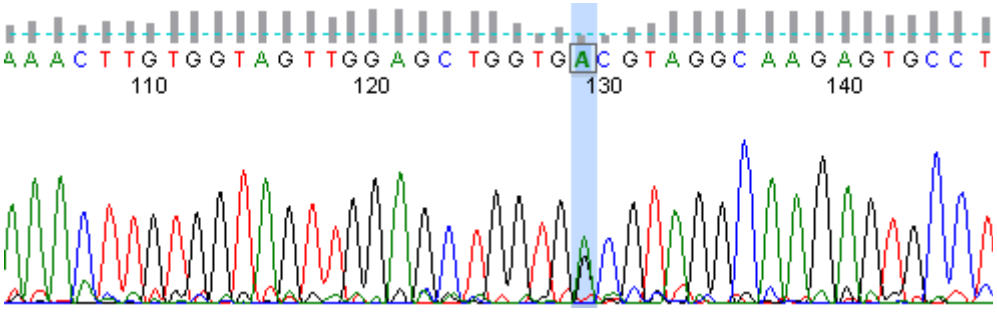
P179 38 G>A



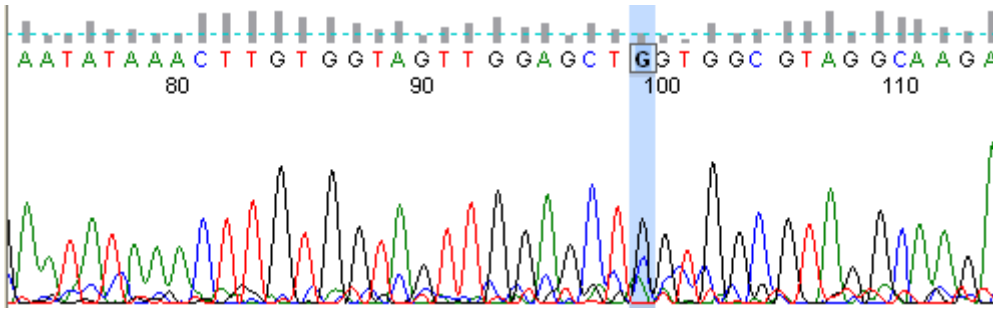
P181 WT



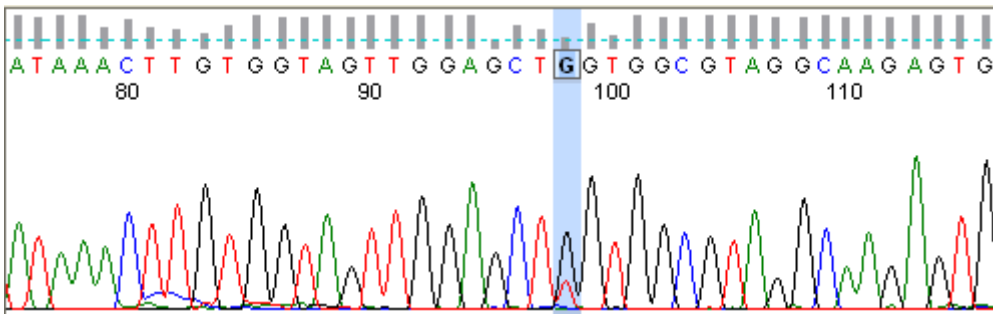
P182 38 G>A



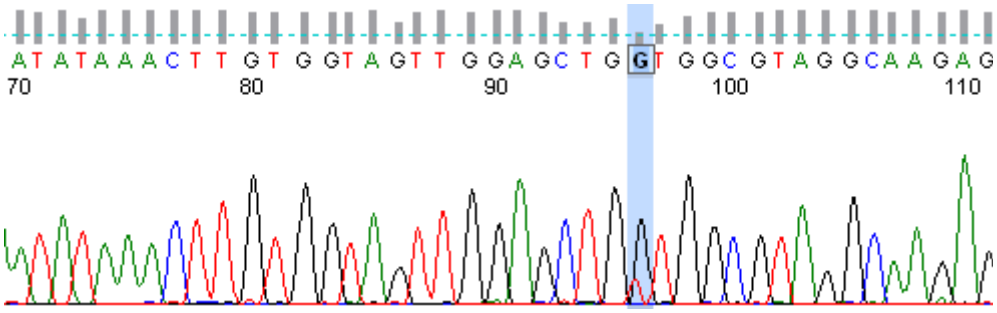
P185 34 G>A



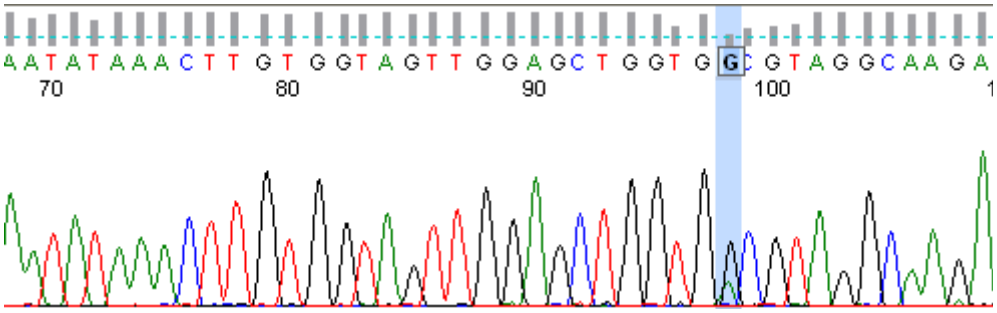
P195 34 G>T



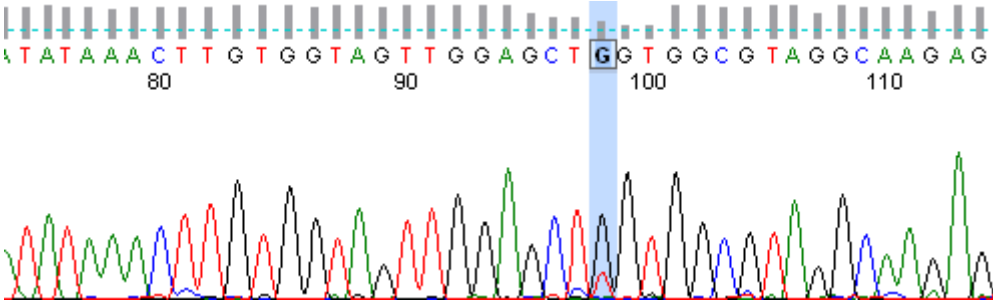
P197 35 G>T



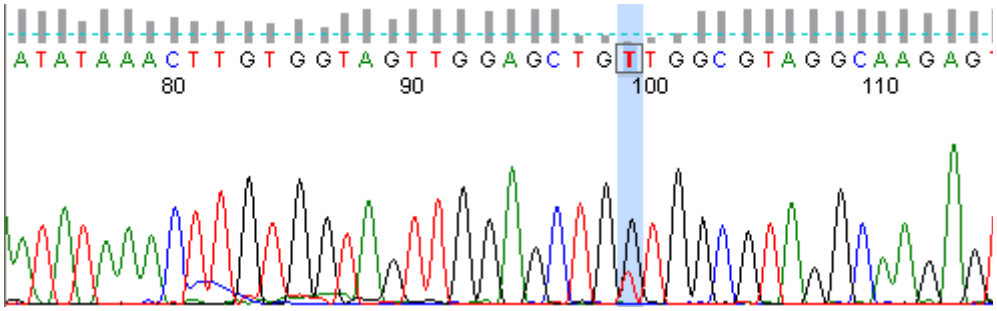
P198 38 G>A



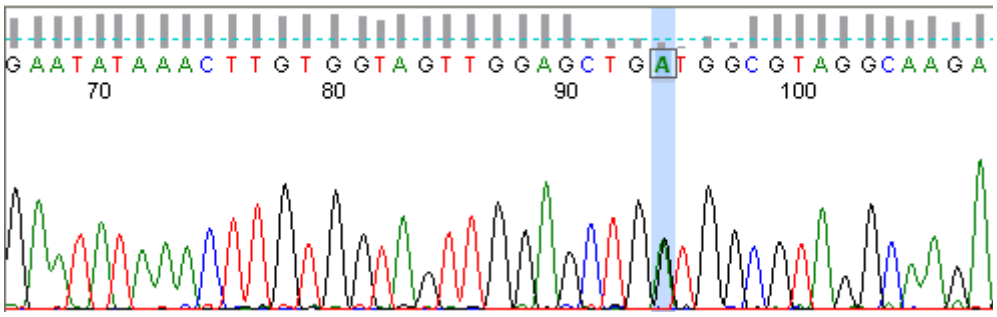
P208 34 G>T



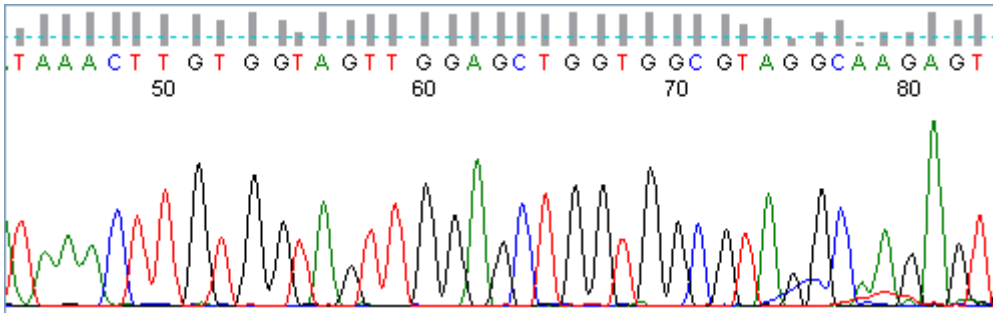
P210 35 G>T



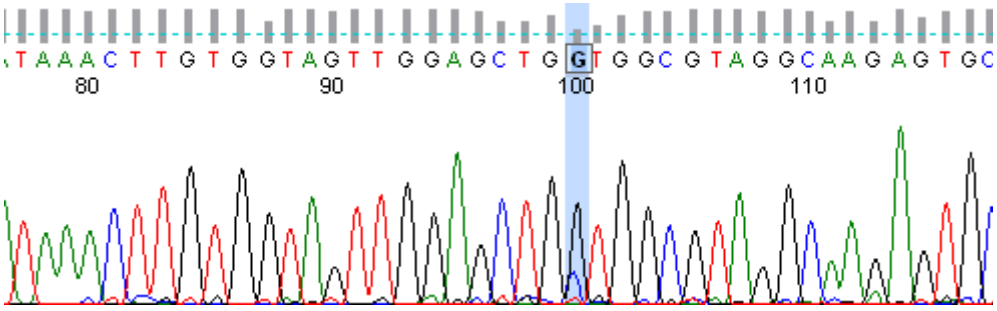
P215 35 G>A



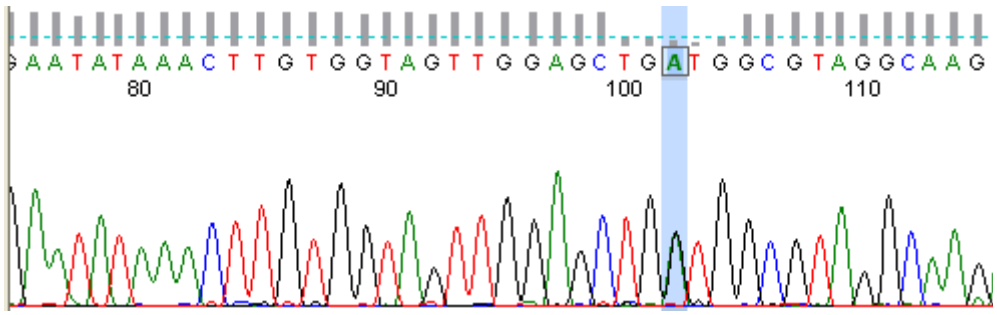
P226 WT; allele-specific PCR: 34 G>T



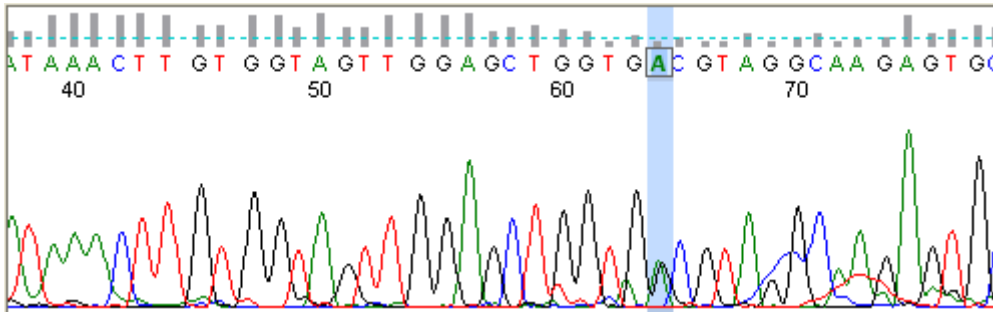
P232 35 G>C



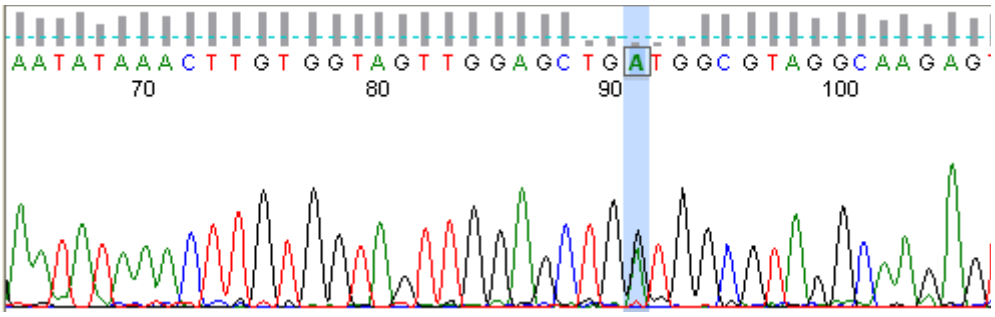
P247 35 G>A



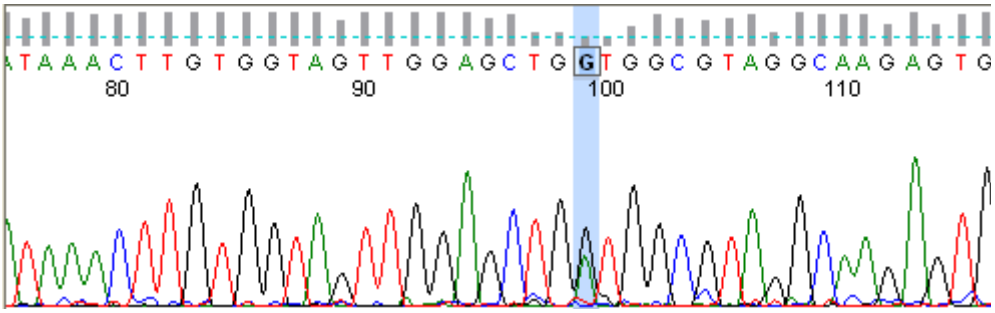
P248 38 G>A



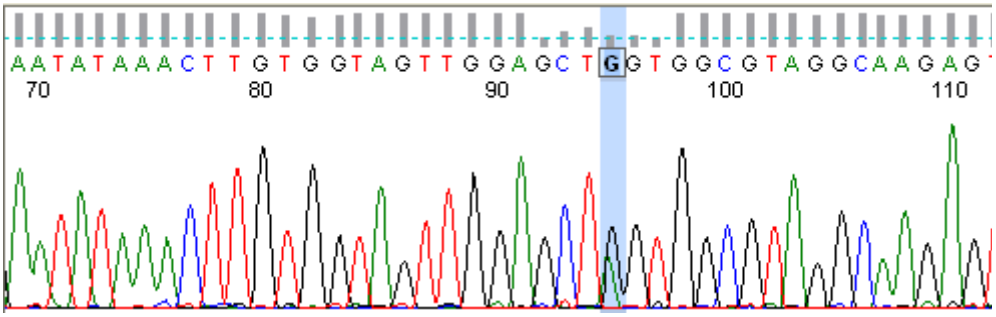
P260 35 G>A



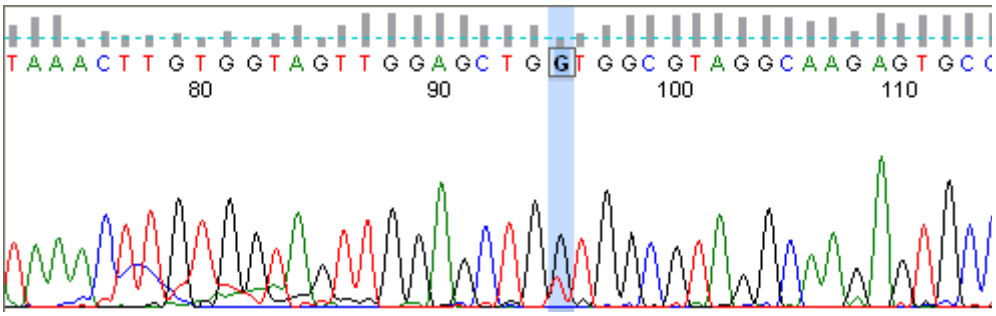
P266 35 G>A



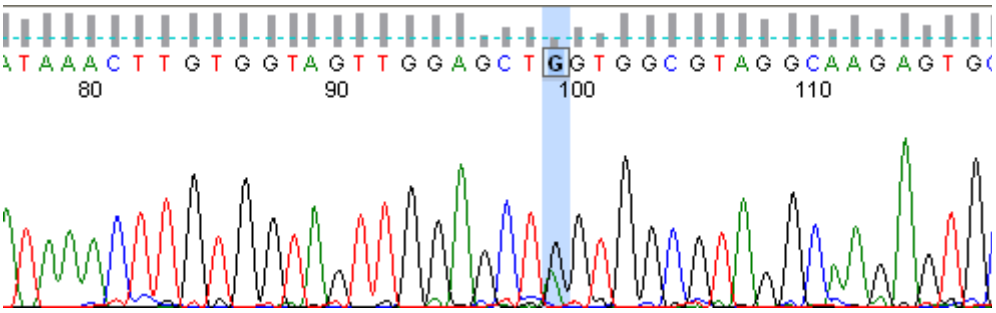
P270 34 G>A



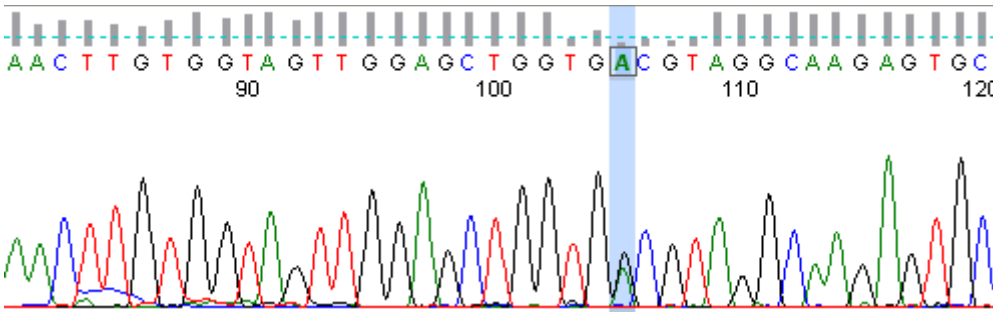
P271 35 G>T



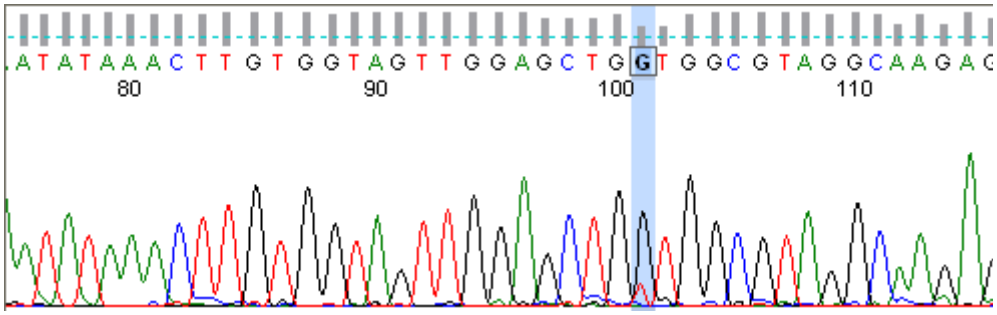
P273 34 G>A



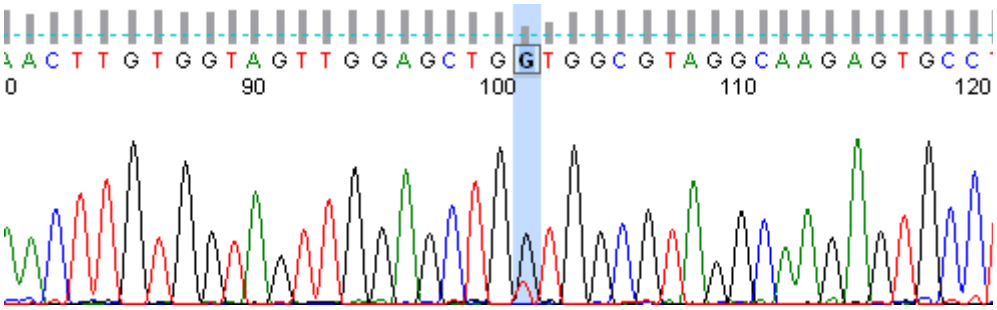
P274 38 G>A



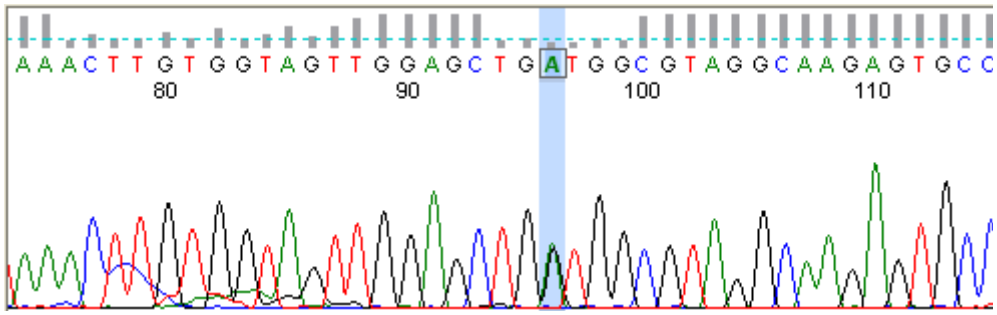
P275 35 G>T



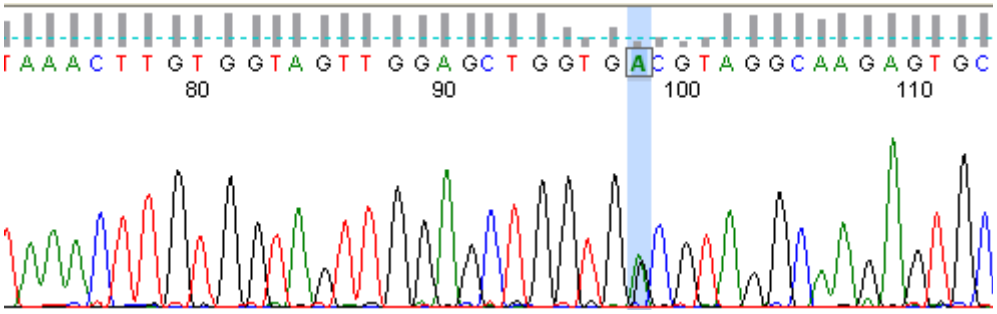
P276 35 G>T



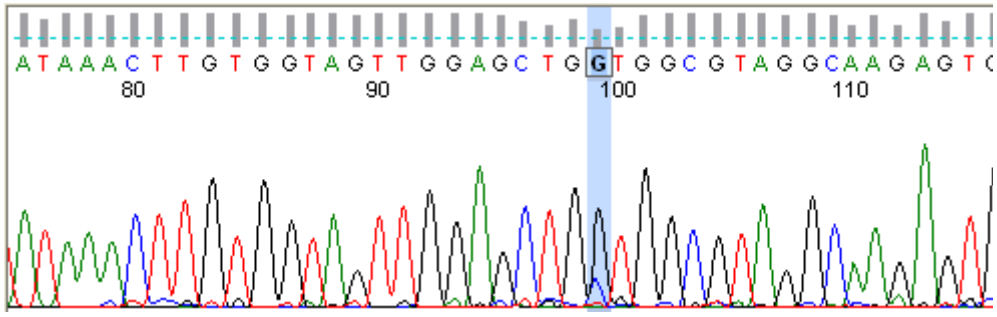
P278 35 G>A



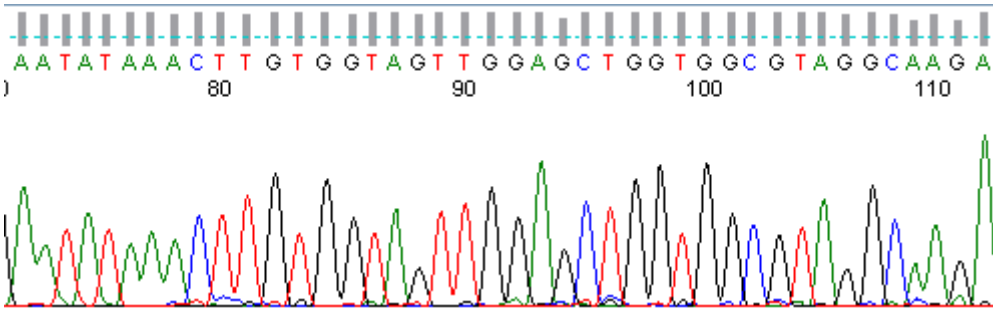
P283 38 G>A



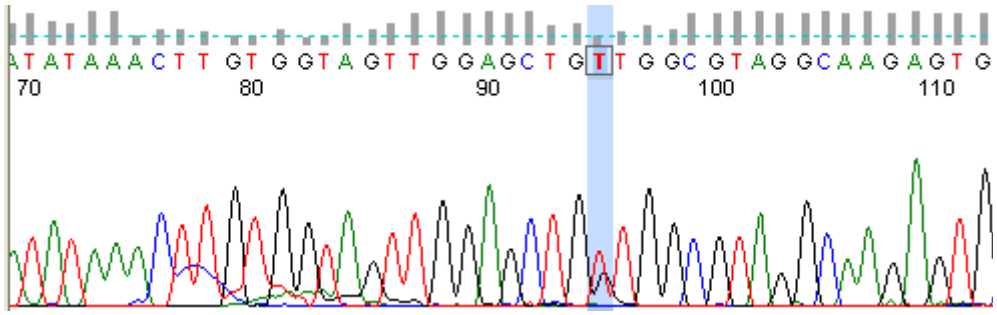
P338 35 G>C



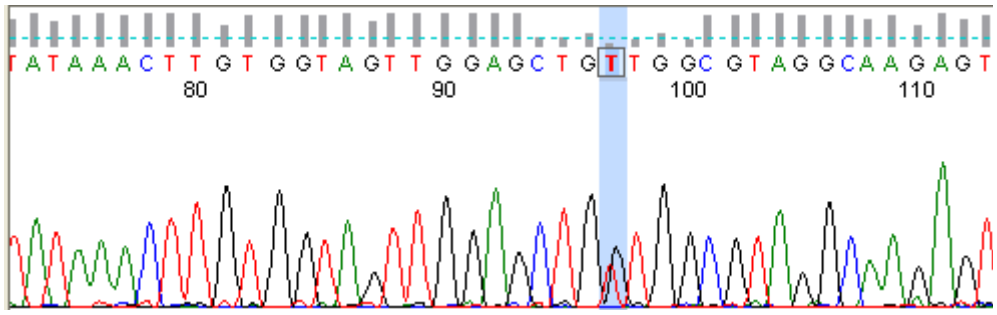
P340 WT



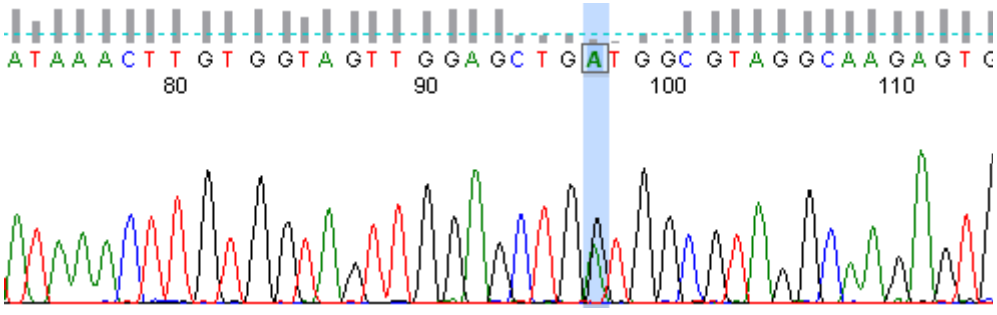
P342 35 G>T



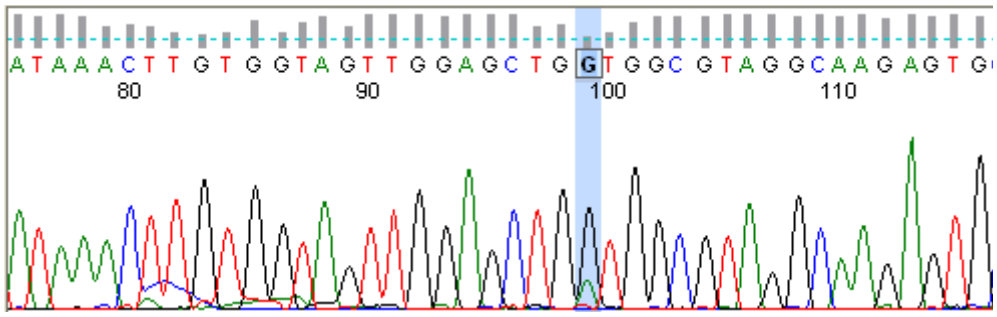
P343 35 G>T



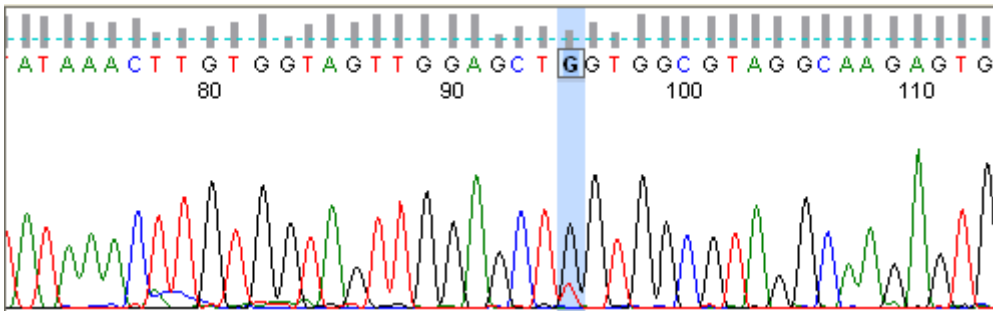
P345 35 G>A



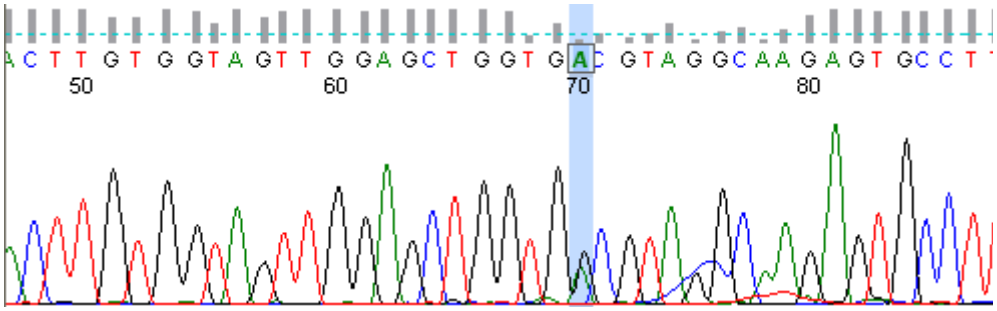
P349 35 G>A



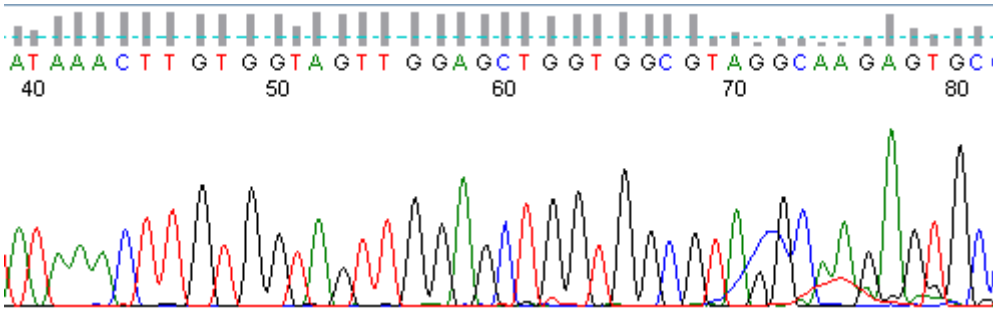
P353 34 G>T



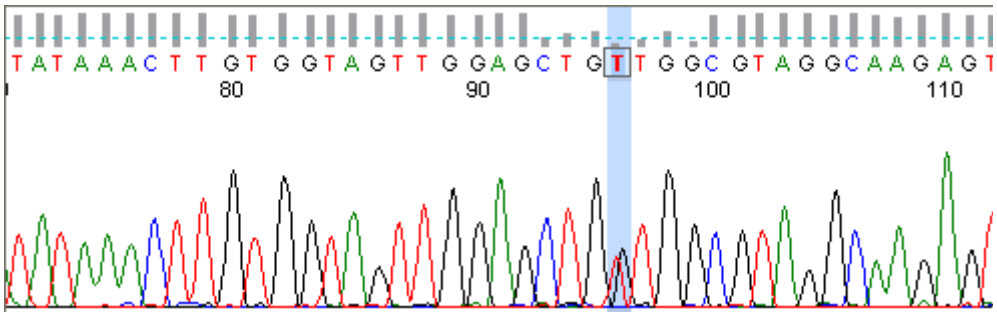
P357 38 G>A



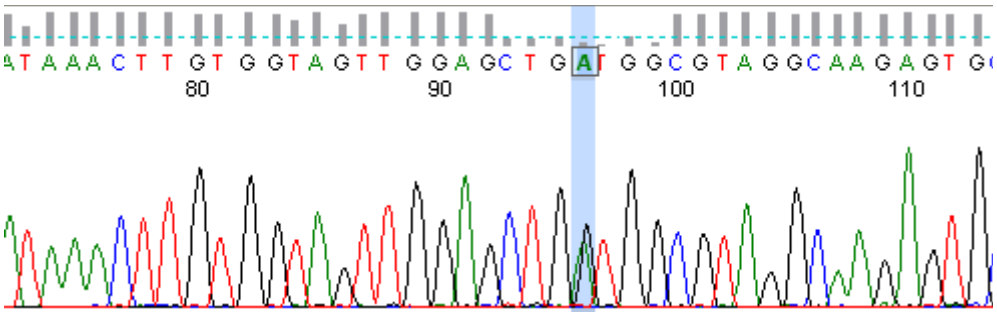
P381 WT; allele-specific PCR: 35 G>A



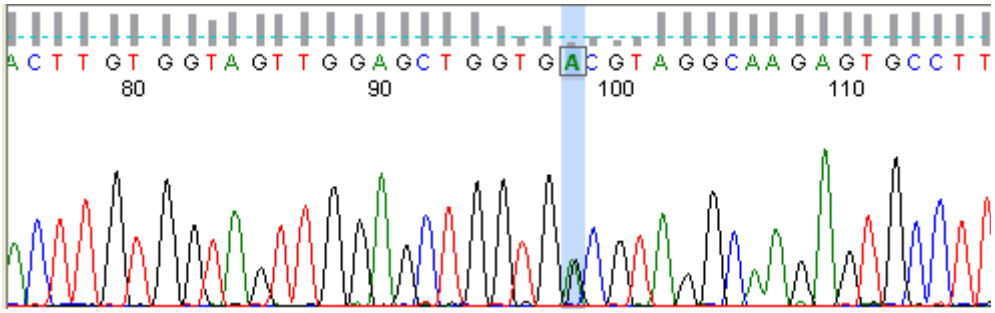
P401 35 G>T



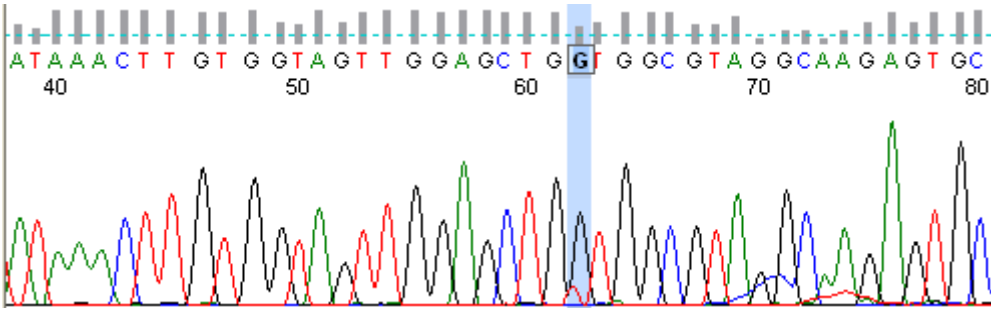
P402 35 G>A



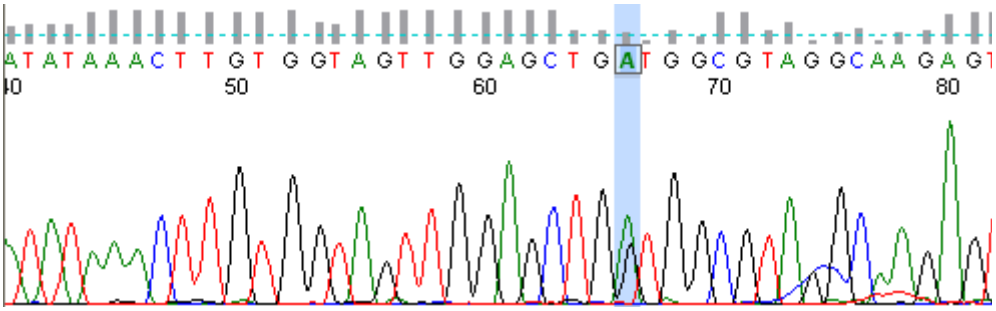
P422 38 G>A



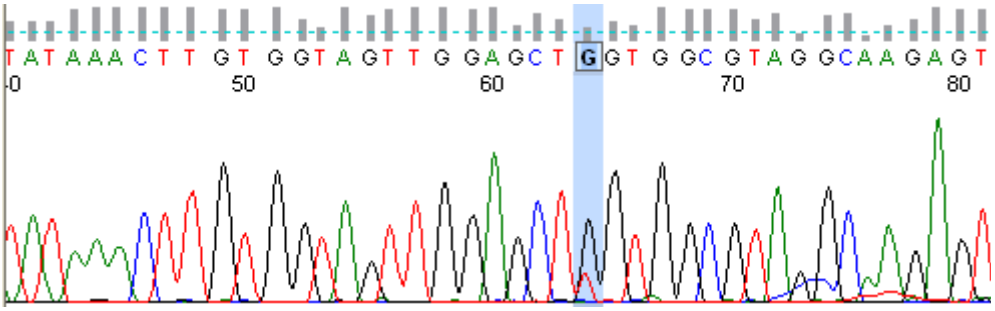
P428 35 G>T



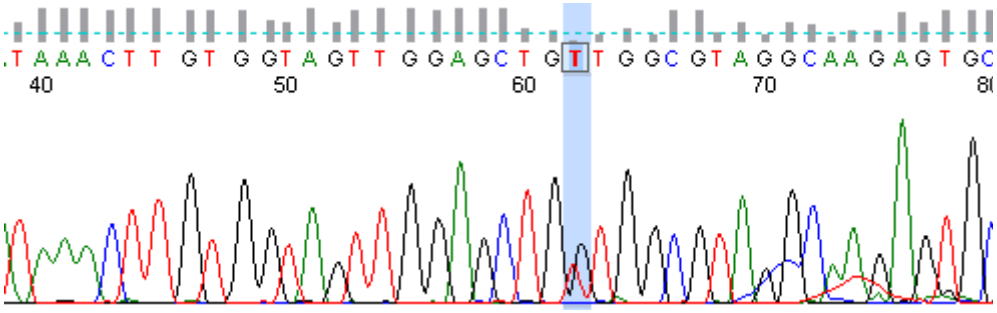
P431 35 G>A



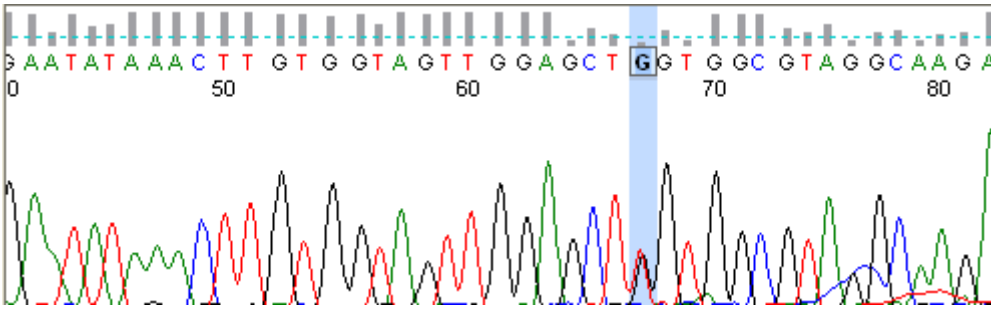
P445 34 G>T



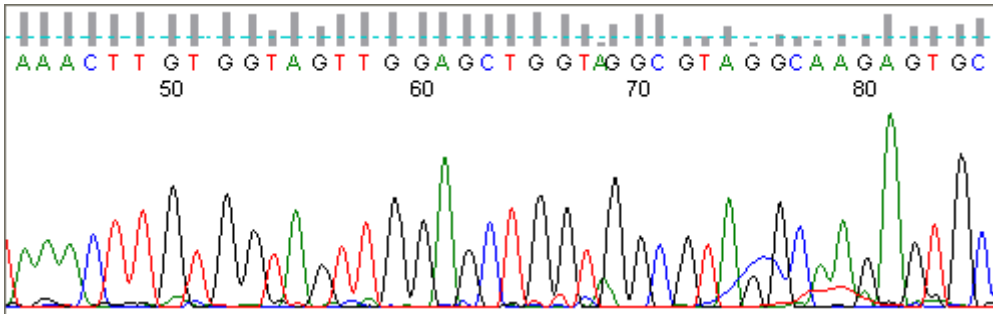
P456 35 G>T



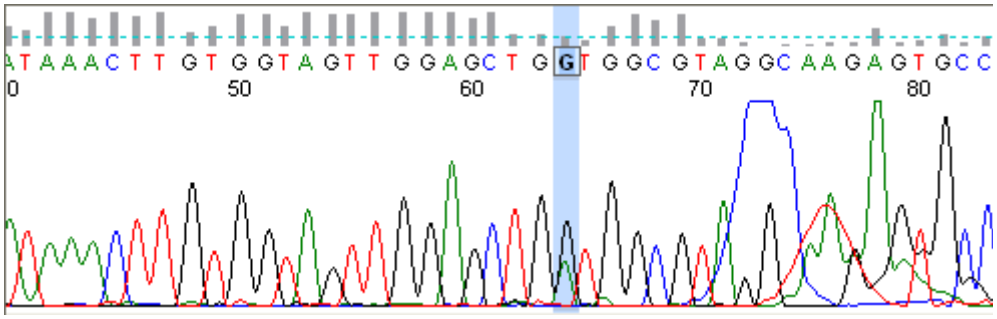
P502 34 G>T



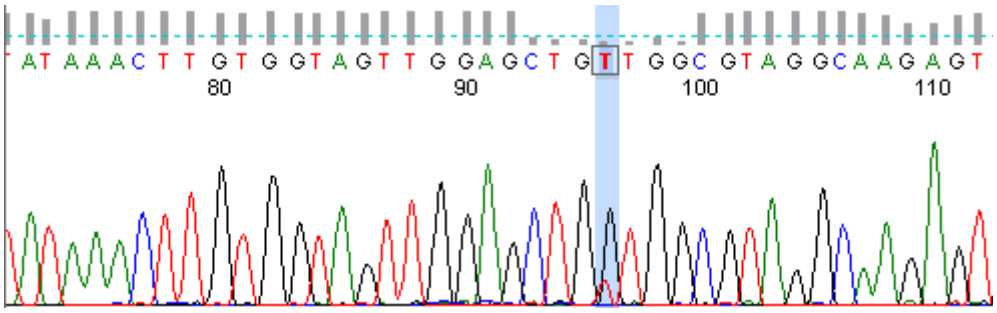
P461 unclear; allele-specific PCR: 35 G>T



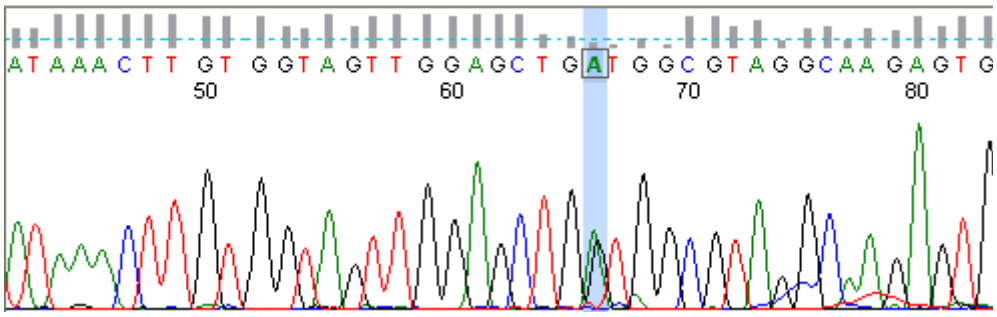
P526 35 G>A



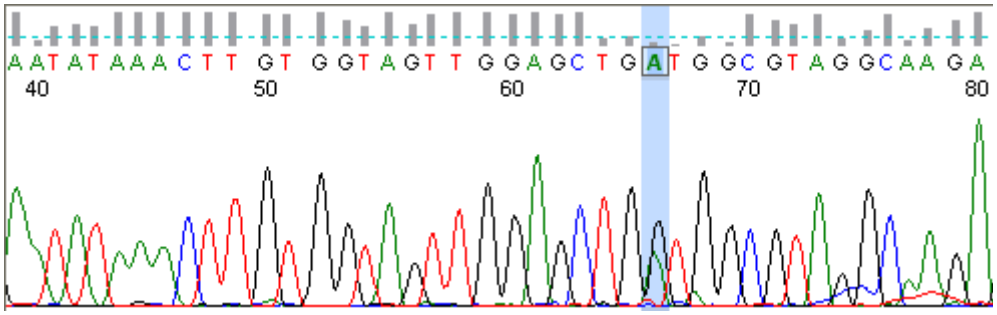
P563 35 G>T



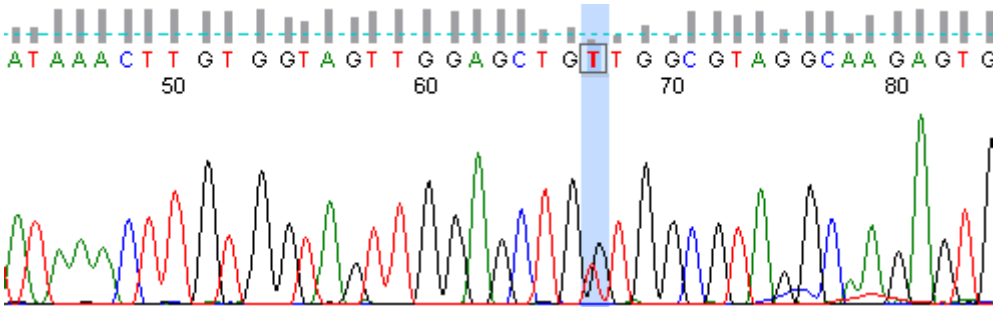
P591 35 G>A



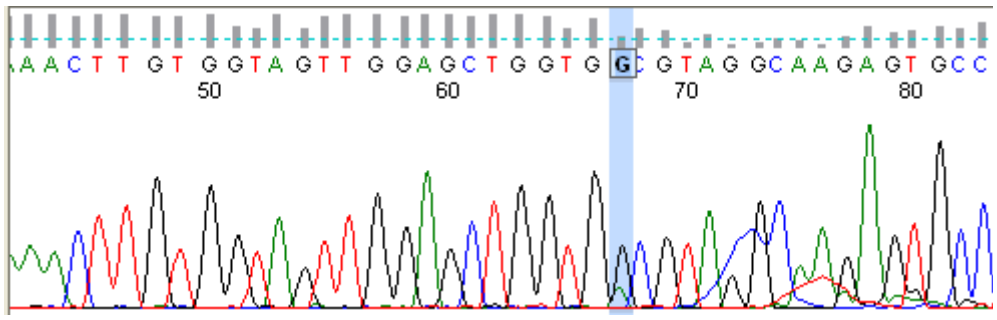
P595 35 G>A



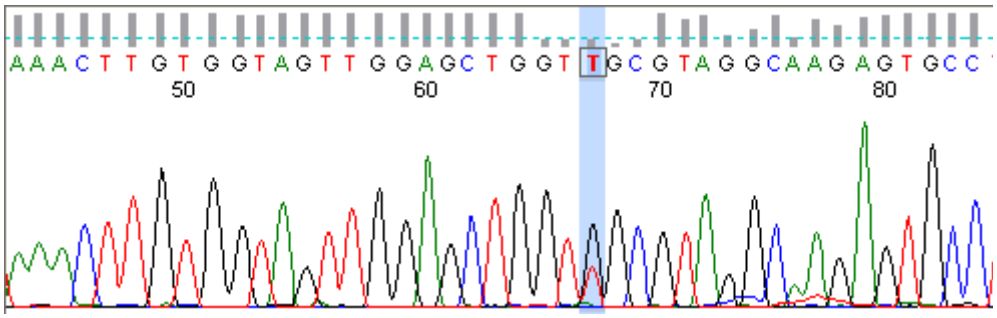
P610 35 G>T



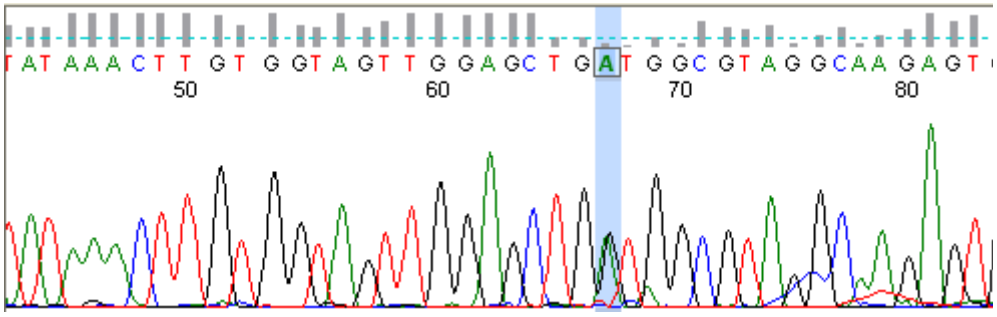
P631 38 G>A



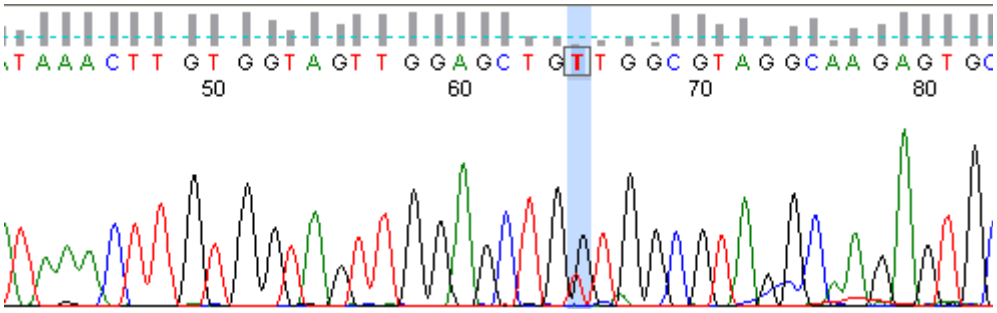
P651 37 G>T



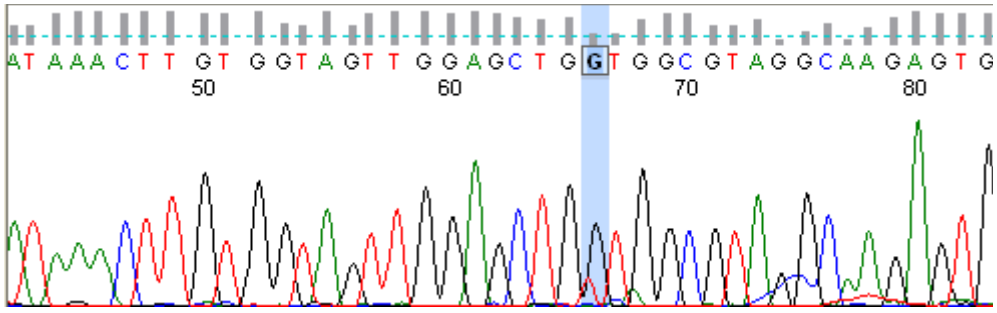
P680 35 G>A



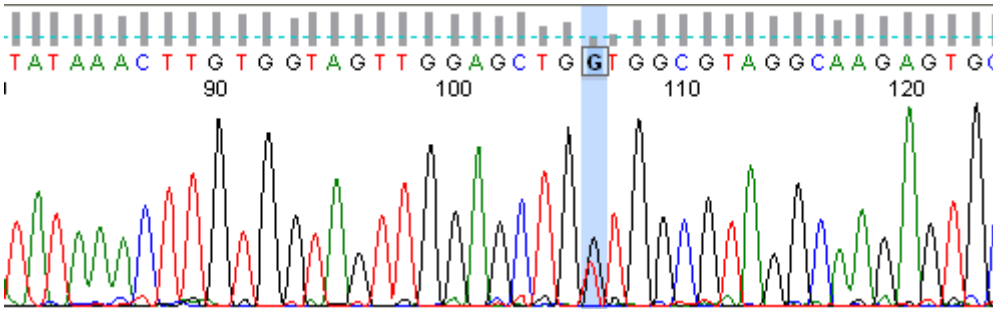
P683 35 G>T



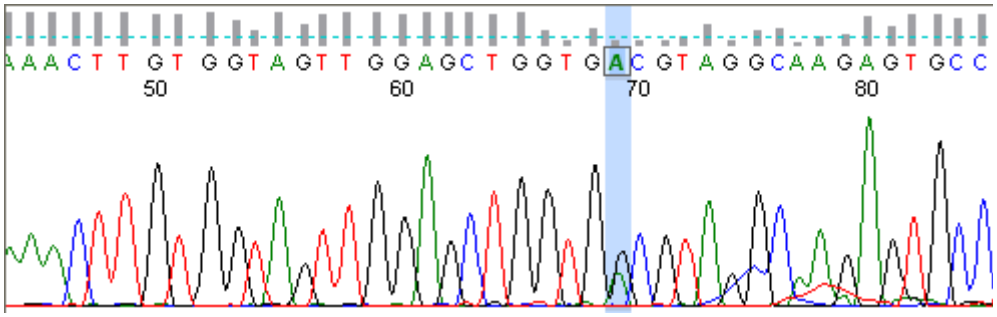
P733 35 G>T



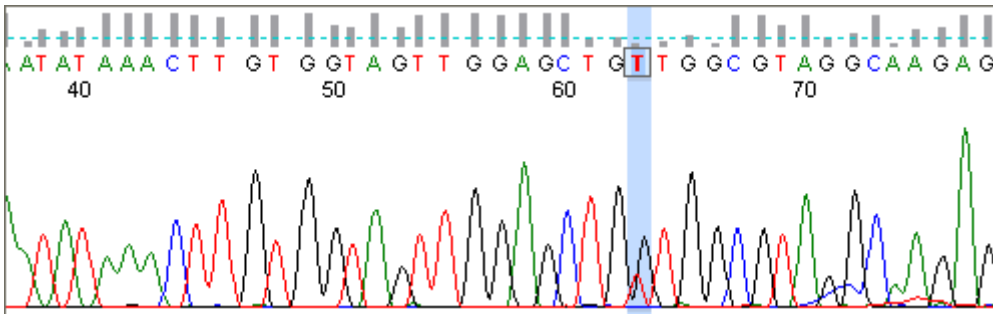
P735 35 G>T



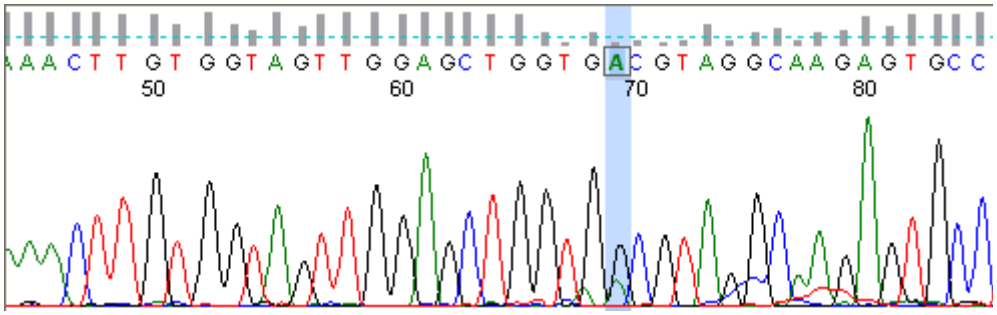
P678 38 G>A



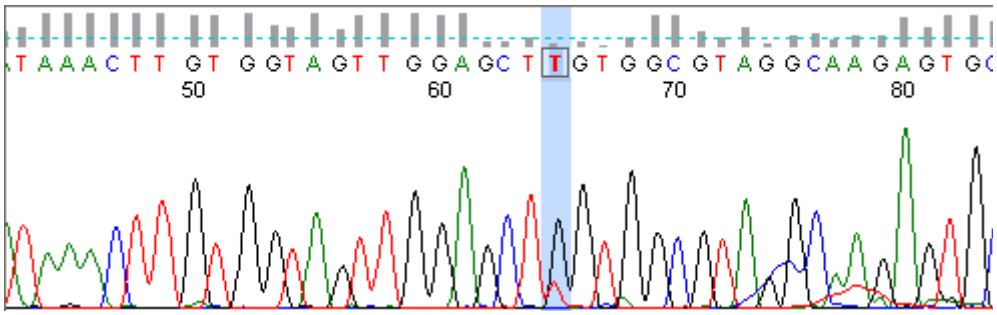
P760 35 G>T



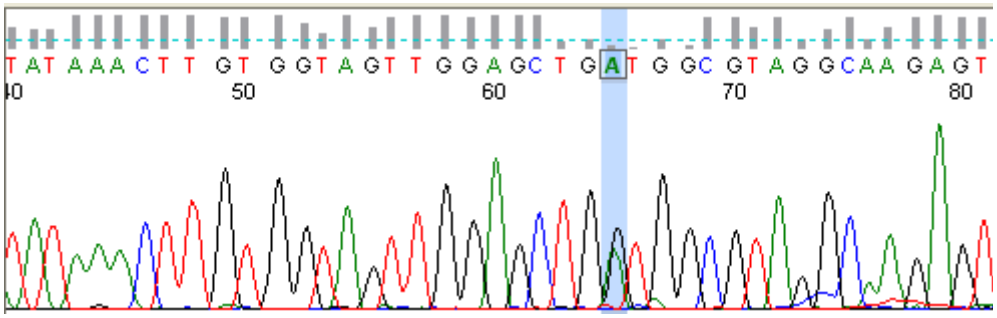
P814 38 G>A



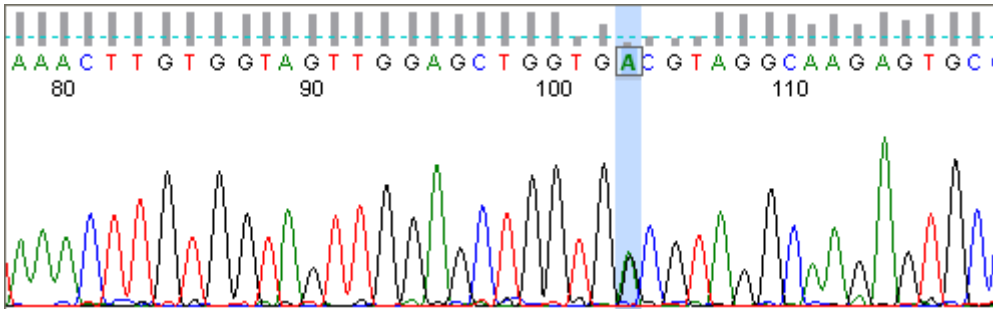
P815 34 G>T



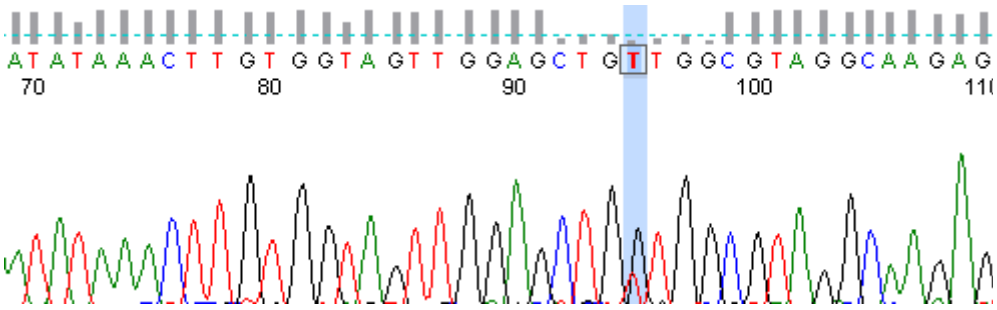
P816 35 G>A



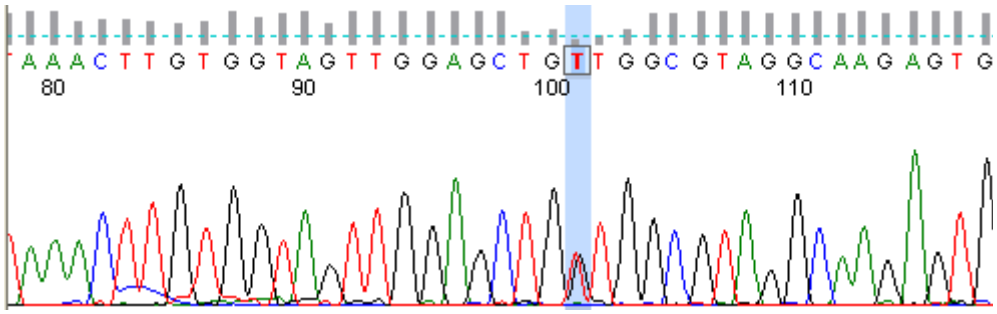
P824 38 G>A



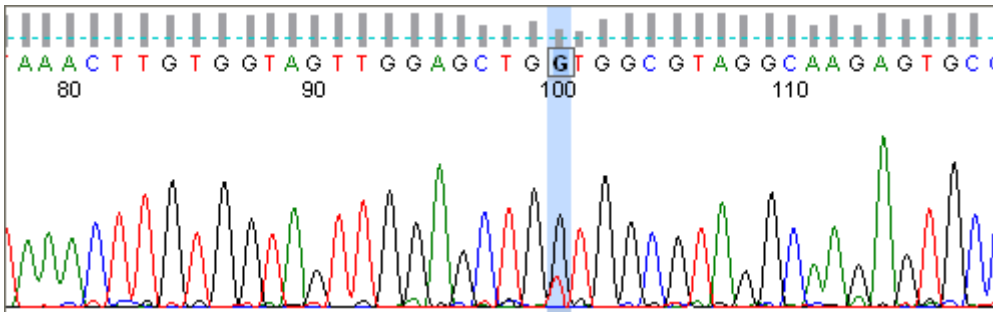
P825 35 G>T



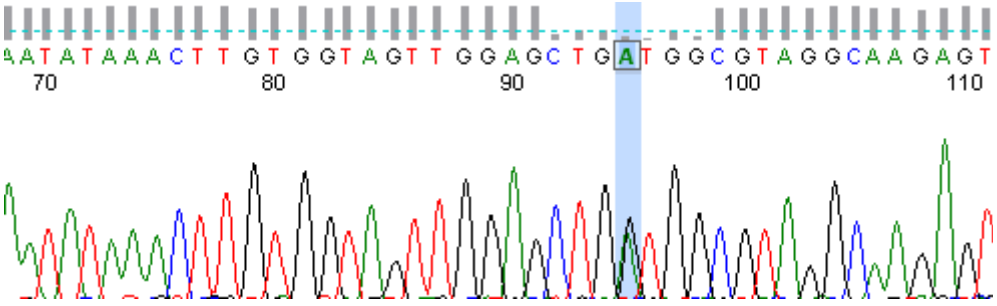
P827 35 G>T



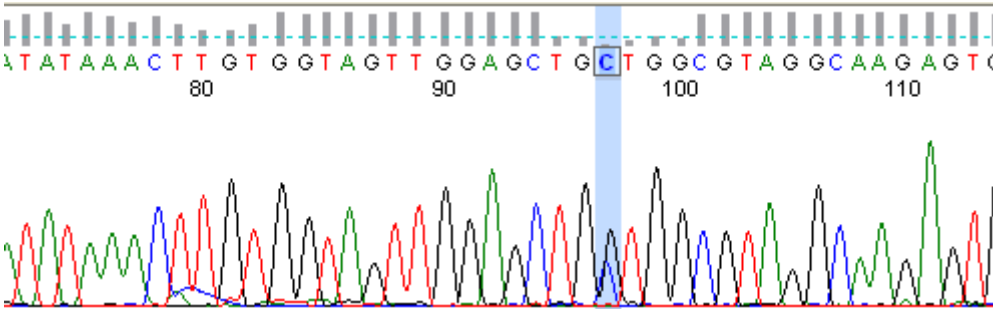
P829 35 G>T



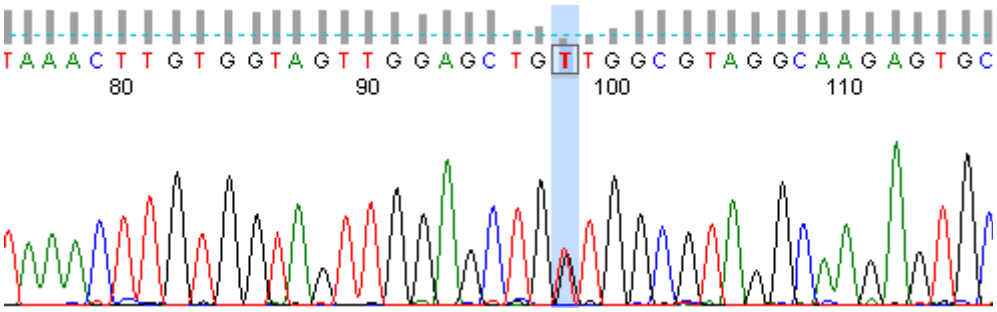
P831 35 G>A



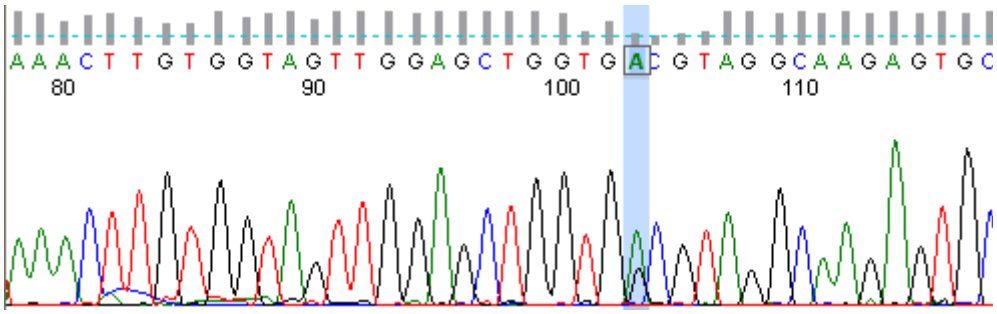
P832 35 G>C



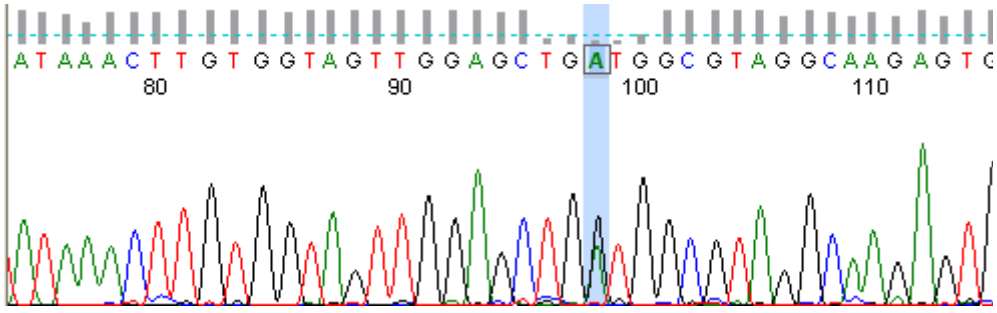
P833 35 G>T



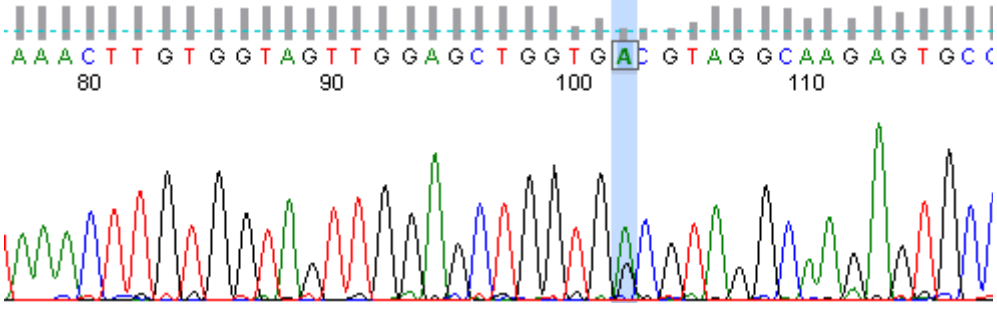
P834 38 G>A



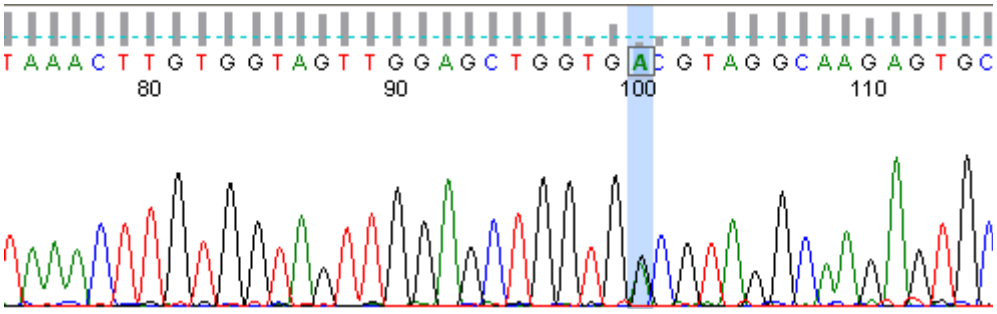
P840 35 G>A



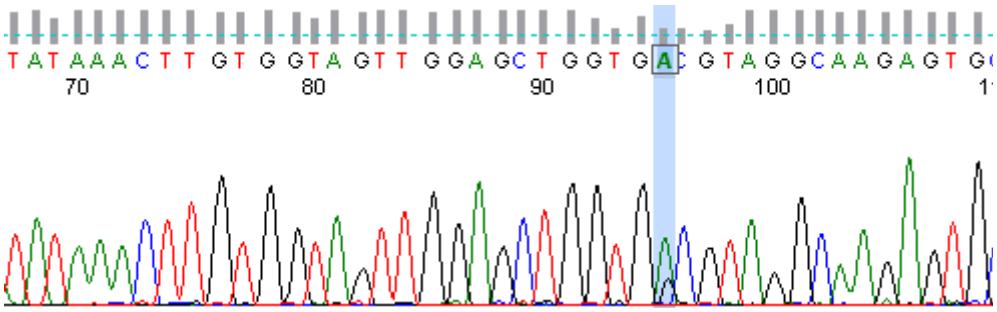
P841 38 G>A



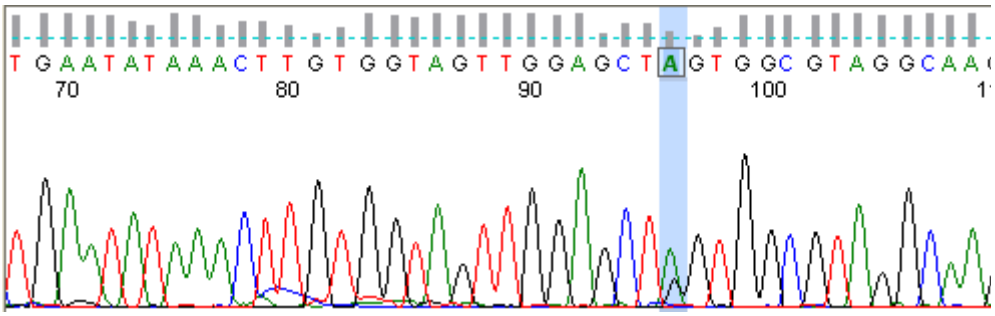
P842 38 G>A



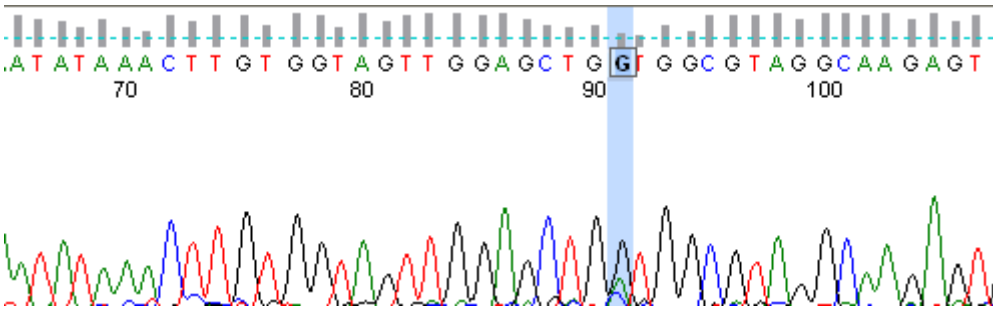
P843 38 G>A



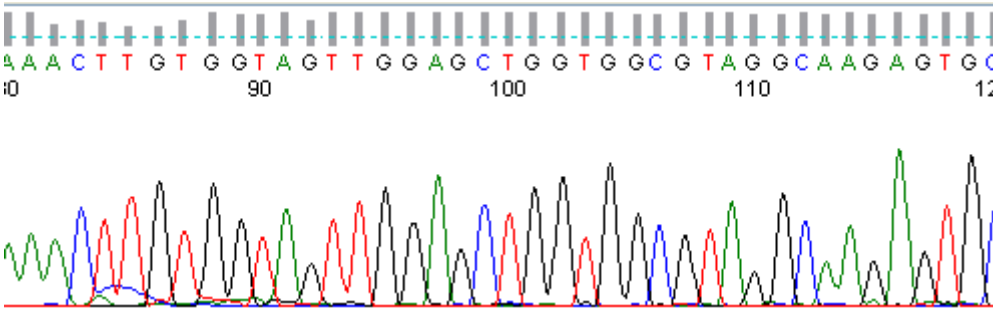
P844 34 G>A



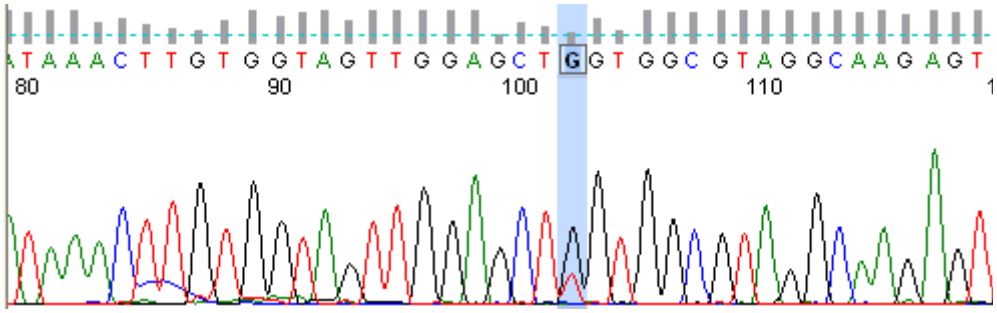
P845 35 G>A



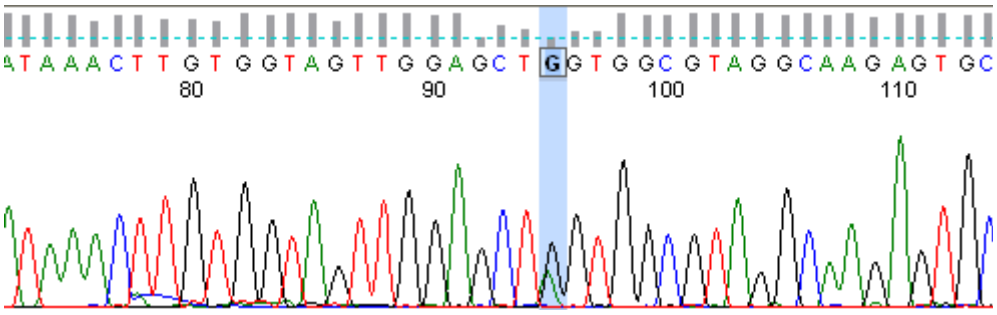
P846 WT



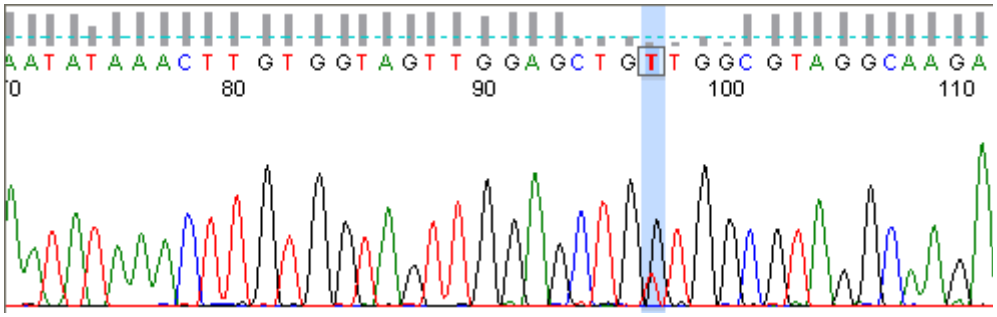
P847 34 G>T



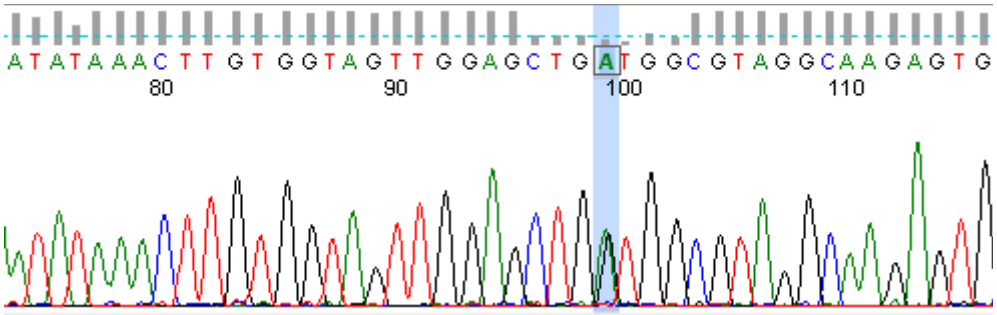
P848 34 G>A



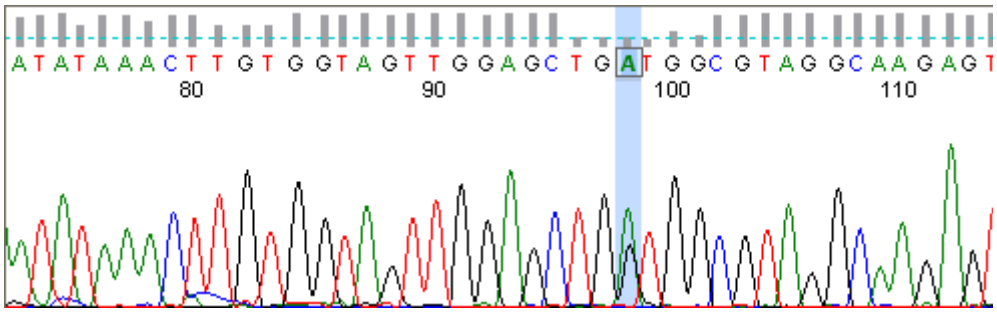
P854 35 G>T



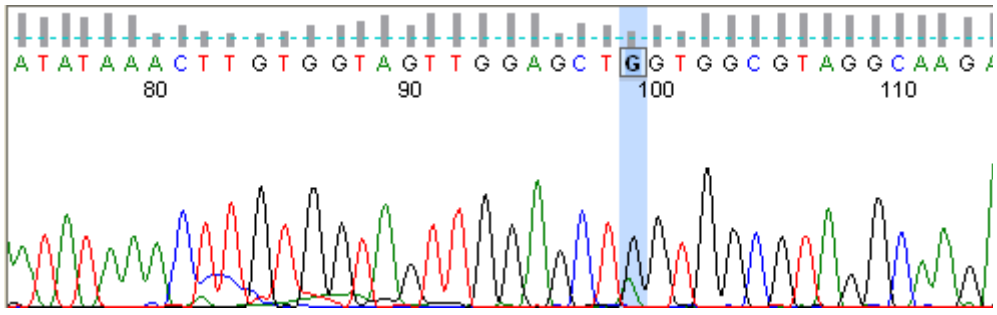
P855 35 G>A



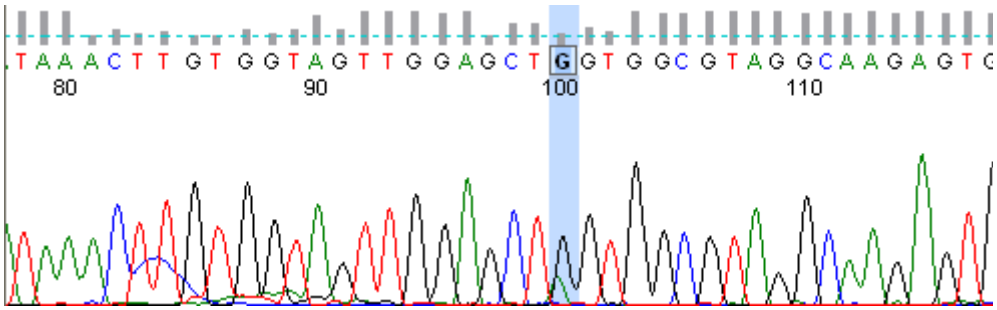
P856 35 G>A



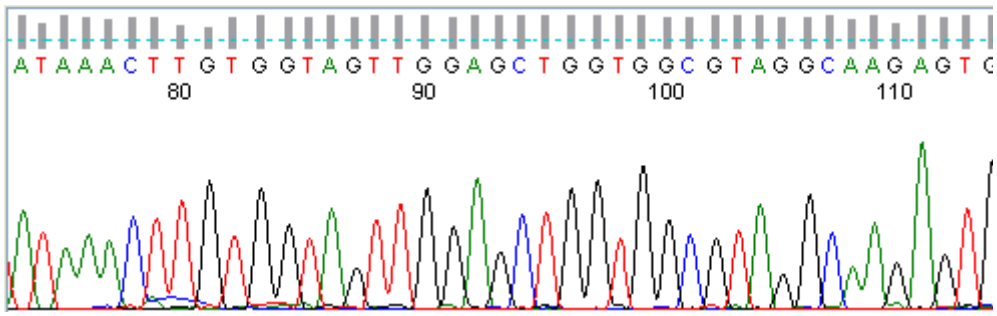
P857 34 G>A



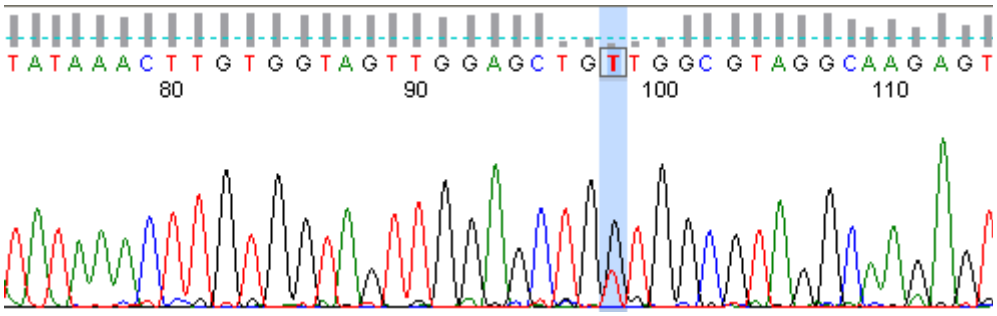
P858 34 G>A



P860 WT



P863 35 G>T



Tab.S7. *Mutation status of KRAS, BRAF, and PIK3CA of the 133 successfully engrafted xenograft models; Mut No- number of detected mutations; Models M110/M111 derived from the same patient. Models M125/M126 also origin from one patient*

No	model-ID	patient-ID	Material type	Mut No	KRAS	BRAF	PIK3CA
1	M25	P592	Snap-frozen Xenograft	1	34 G>A	WT	WT
2	M127	-	Snap-frozen Xenograft	0	WT	WT	WT
3	M19	P575	Snap-frozen Xenograft	1	WT	1799 T>A	WT
4	M132	-	Snap-frozen Xenograft	2	436 G>A	WT	3140 A>G
5	M11	-	Snap-frozen Xenograft	0	WT	WT	WT
6	M120	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
7	M2	-	Snap-frozen Xenograft	0	WT	WT	WT
8	M20	P576	Snap-frozen Xenograft	1	WT	1799 T>A	WT
9	M17	-	Snap-frozen Xenograft	1	38 G>A	WT	WT
10	M121	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
11	M78	P783	Snap-frozen Xenograft	0	WT	WT	WT
12	M22	P583	Snap-frozen Xenograft	0	WT	WT	WT
13	M66	P712	Snap-frozen Xenograft	0	183 A>T	WT	WT
14	M10	P536	Snap-frozen Xenograft	1	WT	1799 T>A	WT
15	M102	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
16	M60	P704	Snap-frozen Xenograft	0	WT	WT	WT
17	M90	-	Snap-frozen Xenograft	2	35 G>T	WT	1633 G>A
18	M1	P494	Snap-frozen Xenograft	0	WT	WT	WT
19	M4	-	Snap-frozen Xenograft	1	436 G>A	WT	WT
20	M95	-	Snap-frozen Xenograft	1	38 G>A	WT	WT
21	M73	P746	Snap-frozen Xenograft	0	WT	WT	WT
22	M14	-	Snap-frozen Xenograft	0	WT	WT	WT
23	M109	-	Snap-frozen Xenograft	0	WT	WT	WT
24	M28	P598	Snap-frozen Xenograft	1	35 G>C	WT	WT
25	M113	-	Snap-frozen Xenograft	0	WT	WT	WT
26	M83	P801	Snap-frozen Xenograft	1	35 G>C	WT	WT
27	M35	-	Snap-frozen Xenograft	1	WT	WT	1633 G>A
28	M104	-	Snap-frozen Xenograft	1	38 G>A	WT	WT
29	M59	P702	Snap-frozen Xenograft	1	WT	1799 T>A	WT
30	M74	P763	Snap-frozen Xenograft	0	WT	WT	WT
31	M30	-	Snap-frozen Xenograft	0	WT	WT	WT
32	M53	-	Snap-frozen Xenograft	1	35 G>A	WT	WT
33	M29	-	Snap-frozen Xenograft	0	WT	WT	WT
34	M128	-	Snap-frozen Xenograft	1	34 G>A	WT	WT
35	M72	-	Snap-frozen Xenograft	0	WT	WT	WT
36	M91	-	Snap-frozen Xenograft	0	WT	WT	WT
37	M88	P812	Snap-frozen Xenograft	0	WT	WT	WT
38	M49	P673	Snap-frozen Xenograft	1	35 G>C	WT	WT
39	M57	P698	Snap-frozen Xenograft	1	38 G>A	WT	WT
40	M130	-	Snap-frozen Xenograft	1	38 G>A	WT	WT
41	M37	P627	Snap-frozen Xenograft	1	WT	WT	1633 G>A
42	M51	P680	Snap-frozen Xenograft	1	35 G>A	WT	WT
43	M13	-	Snap-frozen Xenograft	0	WT	WT	WT
44	M131	-	Snap-frozen Xenograft	0	WT	WT	WT
45	M133	-	Snap-frozen Xenograft	1	436 G>A	WT	WT
46	M114	-	Snap-frozen Xenograft	1	35 G>C	WT	WT
47	M84	-	Snap-frozen Xenograft	0	WT	WT	WT
48	M110	-	Snap-frozen Xenograft	1	182 A>T	WT	1624 G>A

49	M67	P717	Snap-frozen Xenograft	0	WT	WT	WT
50	M36	-	Snap-frozen Xenograft	0	WT	WT	WT
51	M106	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
52	M123	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
53	M76	-	Snap-frozen Xenograft	0	WT	WT	WT
54	M40	P634	Snap-frozen Xenograft	0	WT	WT	WT
55	M101	-	Snap-frozen Xenograft	1	34 G>A, T?	WT	WT
56	M12	-	Snap-frozen Xenograft	2	WT	1799 T>A	WT
57	M48	-	Snap-frozen Xenograft	0	WT	WT	WT
58	M27	P596	Snap-frozen Xenograft	1	35 G>A	WT	WT
59	M99	-	Snap-frozen Xenograft	0	WT	WT	WT
60	M87	P808	Snap-frozen Xenograft	0	WT	WT	WT
61	M118	-	Snap-frozen Xenograft	2	35 G>T	WT	3140 A>G
62	M82	P792	Snap-frozen Xenograft	0	WT	WT	WT
63	M94	-	Snap-frozen Xenograft	0	WT	WT	WT
64	M7	P525	Snap-frozen Xenograft	0	WT	WT	WT
65	M24	P590	Snap-frozen Xenograft	2	35 G>T	WT	1633 G>A
66	M3	P509	Snap-frozen Xenograft	1	35 G>C	WT	WT
67	M68	P733	Snap-frozen Xenograft	1	35 G>T	WT	WT
68	M41	-	Snap-frozen Xenograft	0	WT	WT	WT
69	M26	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
70	M43	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
71	M64	-	Snap-frozen Xenograft	0	WT	WT	WT
72	M42	-	Snap-frozen Xenograft	1	WT	WT	1633 G>A
73	M18	-	Snap-frozen Xenograft	1	35 G>A	WT	WT
74	M44	-	Snap-frozen Xenograft	0	WT	WT	WT
75	M77	-	Snap-frozen Xenograft	0	WT	WT	WT
76	M115	-	Snap-frozen Xenograft	1	38 G>A	WT	WT
77	M9	-	Snap-frozen Xenograft	2	38 G>A	WT	1633 G>A
78	M96	-	Snap-frozen Xenograft	2	35 G>T	WT	1633 G>A
79	M75	-	Snap-frozen Xenograft	1	35 G>C	WT	WT
80	M81	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
81	M58	-	Snap-frozen Xenograft	1	WT	WT	1633 G>A
82	M63	-	Snap-frozen Xenograft	1	38 G>A	WT	WT
83	M5	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
84	M6	-	Snap-frozen Xenograft	0	WT	WT	WT
85	M122	-	Snap-frozen Xenograft	0	WT	WT	WT
86	M107	-	Snap-frozen Xenograft	1	38 G>A	WT	WT
87	M23	-	Snap-frozen Xenograft	0	WT	WT	WT
88	M32	-	Snap-frozen Xenograft	2	WT	1799 T>A	1633 G>A
89	M100	-	Snap-frozen Xenograft	0	WT	WT	WT
90	M97	-	Snap-frozen Xenograft	0	WT	WT	WT
91	M31	-	Snap-frozen Xenograft	2	38 G>A	WT	3140 A>G
92	M119	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
93	M86	-	Snap-frozen Xenograft	1	35 G>A	WT	WT
94	M56	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
95	M62	-	Snap-frozen Xenograft	0	WT	WT	WT
96	M103	-	Snap-frozen Xenograft	0	WT	WT	WT
97	M89	-	Snap-frozen Xenograft	0	WT	WT	WT
98	M34	-	Snap-frozen Xenograft	0	WT	WT	WT
99	M33	-	Snap-frozen Xenograft	1	WT	1799 T>A	WT
100	M45	-	Snap-frozen Xenograft	2	WT	1799 T>A	1633 G>A
101	M70	-	Snap-frozen Xenograft	0	WT	WT	WT
102	M93	-	Snap-frozen Xenograft	0	WT	WT	WT

103	M65	-	Snap-frozen Xenograft	2	436 G>A	WT	1633 G>A
104	M8	-	Snap-frozen Xenograft	0	WT	WT	WT
105	M16	-	Snap-frozen Xenograft	0	WT	WT	WT
106	M79	-	Snap-frozen Xenograft	0	WT	WT	WT
107	M46	-	Snap-frozen Xenograft	0	WT	WT	WT
108	M69	-	Snap-frozen Xenograft	1	WT	WT	1624 G>A
109	M105	-	Snap-frozen Xenograft	0	WT	WT	WT
110	M125	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
111	M129	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
112	M116	-	Snap-frozen Xenograft	0	WT	WT	WT
113	M108	-	Snap-frozen Xenograft	0	WT	WT	WT
114	M80	-	Snap-frozen Xenograft	0	WT	WT	WT
115	M54	-	Snap-frozen Xenograft	0	WT	WT	WT
116	M92	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
117	M52	-	Snap-frozen Xenograft	1	35 G>C	WT	WT
118	M50	-	Snap-frozen Xenograft	0	WT	WT	WT
119	M71	-	Snap-frozen Xenograft	0	WT	WT	WT
120	M47	-	Snap-frozen Xenograft	1	35 G>C	WT	WT
121	M124	-	Snap-frozen Xenograft	0	WT	WT	WT
122	M117	-	Snap-frozen Xenograft	1	WT	WT	1633 G>A
123	M55	-	Snap-frozen Xenograft	0	WT	WT	WT
124	M98	-	Snap-frozen Xenograft	0	WT	WT	WT
125	M61	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
126	M39	-	Snap-frozen Xenograft	0	WT	WT	WT
127	M112	-	Snap-frozen Xenograft	0	WT	WT	WT
128	M14	-	Snap-frozen Xenograft	1	35 G>A	WT	WT
129	M85	-	Snap-frozen Xenograft	1	35 G>A	WT	WT
130	M38	-	Snap-frozen Xenograft	0	WT	WT	WT
131	M21	-	Snap-frozen Xenograft	1	WT	1799 T>A	NA
132	M126	-	Snap-frozen Xenograft	1	35 G>T	WT	WT
133	M111	-	Snap-frozen Xenograft	1	182 A>T	WT	1624 G>A

Tab.S8. *Median Ct values indicating expression of the selected genes analyzed in the RNA isolated from the tissue of the xenograft, primary snap-frozen tumours, and primary FFPE tissues. HK - housekeeping genes (GAPDH, RPLP0, UBC)which mean of the median Ct values was substracted in order to normalized expression data*

Model/Analyzed Gene			**Xenograft Frozen**			**Corresponding Primary Frozen**					
No	**model-ID**	**Detector**	**Ct Median**	**Ct HK**	**Ct Median - HK Medians**	**Ct**	**Ct HK Medians**	**Ct Median - HK**	**Ct Median**	**Ct HK**	**Ct Median - HK**
1	M1	AREG	28,86	24,67	4,19	24,95	19,86	5,08	30,31	27,60	2,71
2	M1	EREG	33,85	24,67	9,18	28,26	19,86	8,39	35,51	27,60	7,92
3	M1	DUSP6	-	-	-	31,96	19,86	12,10	-	-	-
4	M1	SLC26A3	-	-	-	23,57	19,86	3,71	-	-	-
5	M1	PTPRF	-	-	-	25,09	19,86	5,23	-	-	-
6	M1	LOC158960	-	-	-	31,34	19,86	11,48	-	-	-
7	M1	PTEN	34,32	24,67	9,65	-	-	-	-	-	-
8	M1	GAPDH	24,29	24,67	-0,38	18,96	19,86	-0,91	27,33	27,60	-0,27
9	M1	RPLP0	22,98	24,67	-1,70	19,32	19,86	-0,55	26,34	27,60	-1,26
10	M1	UBC	26,75	24,67	2,08	21,32	19,86	1,46	24,04	27,60	-3,56
11	M18	AREG	30,09	23,11	6,98	31,28	24,04	7,25	35,28	28,38	6,90
12	M18	EREG	34,13	23,11	11,03	36,29	24,04	12,26	39,62	28,38	11,24
13	M18	DUSP6	-	-	-	33,75	24,04	9,71	-	-	-
14	M18	SLC26A3	-	-	-	31,18	24,04	7,14	-	-	-
15	M18	PTPRF	-	-	-	28,07	24,04	4,04	-	-	-
16	M18	LOC158960	-	-	-	36,23	24,04	12,20	-	-	-
17	M18	PTEN	34,09	23,11	10,98	-	-	-	-	-	-
18	M18	GAPDH	22,40	23,11	-0,71	24,13	24,04	0,10	28,03	28,38	-0,35
19	M18	RPLP0	21,31	23,11	-1,80	22,94	24,04	-1,10	27,25	28,38	-1,13
20	M18	UBC	25,62	23,11	2,51	25,04	24,04	1,01	29,86	28,38	1,48
21	M27	AREG	28,28	24,26	4,02	25,86	21,23	4,63	31,01	27,44	3,57
22	M27	EREG	33,86	24,26	9,60	29,91	21,23	8,68	38,18	27,44	10,74
23	M27	DUSP6	-	-	-	30,50	21,23	9,27	-	-	-
24	M27	SLC26A3	-	-	-	29,12	21,23	7,89	-	-	-
25	M27	PTPRF	-	-	-	26,32	21,23	5,09	-	-	-
26	M27	LOC158960	-	-	-	34,35	21,23	13,12	-	-	-
27	M27	PTEN	35,30	24,26	11,04	-	-	-	-	-	-
28	M27	GAPDH	23,27	24,26	-1,00	19,65	21,23	-1,58	26,51	27,44	-0,93
29	M27	RPLP0	22,12	24,26	-2,14	20,55	21,23	-0,68	26,35	27,44	-1,09
30	M27	UBC	27,40	24,26	3,14	23,50	21,23	2,27	29,46	27,44	2,02
31	M52	AREG	27,59	23,23	4,35	25,48	22,40	3,08	37,49	32,90	4,59

32	M52	EREG	32,11	23,23	8,88	30,67	22,40	8,27	40,00	32,90	7,10
33	M52	DUSP6	-	-	-	31,87	22,40	9,48	-	-	-
34	M52	SLC26A3	-	-	-	29,26	22,40	6,87	-	-	-
35	M52	PTPRF	-	-	-	25,73	22,40	3,34	-	-	-
36	M52	LOC158960	-	-	-	34,53	22,40	12,13	-	-	-
37	M52	PTEN	34,05	23,23	10,81	-	-	-	-	-	-
38	M52	GAPDH	22,63	23,23	-0,61	21,96	22,40	-0,43	34,82	32,90	1,92
39	M52	RPLP0	22,05	23,23	-1,18	21,86	22,40	-0,54	30,98	32,90	-1,92
40	M52	UBC	25,03	23,23	1,79	23,36	22,40	0,97	-	32,90	-
41	M55	AREG	27,16	23,21	3,95	25,23	20,53	4,70	33,78	28,09	5,69
42	M55	EREG	30,05	23,21	6,84	26,85	20,53	6,32	35,21	28,09	7,12
43	M55	DUSP6	-	-	-	31,96	20,53	11,43	-	-	-
44	M55	SLC26A3	-	-	-	23,76	20,53	3,23	-	-	-
45	M55	PTPRF	-	-	-	25,18	20,53	4,65	-	-	-
46	M55	LOC158960	-	-	-	31,62	20,53	11,09	-	-	-
47	M55	PTEN	36,09	23,21	12,88	-	-	-	-	-	-
48	M55	GAPDH	22,50	23,21	-0,71	19,39	20,53	-1,14	28,47	28,09	0,38
49	M55	RPLP0	22,67	23,21	-0,54	20,14	20,53	-0,39	27,71	28,09	-0,38
50	M55	UBC	24,47	23,21	1,26	22,06	20,53	1,53	-	28,09	-
51	M59	AREG	30,37	23,03	7,34	27,95	21,88	6,08	34,00	25,76	8,24
52	M59	EREG	36,10	23,03	13,06	31,39	21,88	9,51	37,07	25,76	11,31
53	M59	DUSP6	-	-	-	31,28	21,88	9,40	-	-	-
54	M59	SLC26A3	-	-	-	33,22	21,88	11,34	-	-	-
55	M59	PTPRF	-	-	-	27,33	21,88	5,46	-	-	-
56	M59	LOC158960	-	-	-	32,94	21,88	11,06	-	-	-
57	M59	PTEN	33,02	23,03	9,98	-	-	-	-	-	-
58	M59	GAPDH	23,24	23,03	0,20	22,73	21,88	0,86	26,46	25,76	0,70
59	M59	RPLP0	21,43	23,03	-1,60	19,38	21,88	-2,50	25,06	25,76	-0,70
60	M59	UBC	24,43	23,03	1,40	23,52	21,88	1,64	-	25,76	-
61	M63	AREG	31,31	23,06	8,25	26,63	20,61	6,02	34,17	27,78	6,39
62	M63	EREG	35,51	23,06	12,45	31,17	20,61	10,56	39,41	27,78	11,62
63	M63	DUSP6	-	-	-	31,63	20,61	11,01	-	-	-
64	M63	SLC26A3	-	-	-	26,65	20,61	6,04	-	-	-
65	M63	PTPRF	-	-	-	27,29	20,61	6,68	-	-	-
66	M63	LOC158960	-	-	-	33,10	20,61	12,49	-	-	-
67	M63	PTEN	33,36	23,06	10,30	-	-	-	-	-	-
68	M63	GAPDH	22,60	23,06	-0,46	20,20	20,61	-0,41	27,20	27,78	-0,58
69	M63	RPLP0	21,12	23,06	-1,94	19,09	20,61	-1,52	26,58	27,78	-1,20

70	M63	UBC	25,45	23,06	2,40	22,54	20,61	1,93	29,56	27,78	1,78
71	M65	AREG	33,37	26,89	6,48	25,23	21,91	3,32	31,00	27,39	3,61
72	M65	EREG	37,88	26,89	10,99	29,82	21,91	7,91	34,18	27,39	6,79
73	M65	DUSP6	-	-	-	32,62	21,91	10,71	-	-	-
74	M65	SLC26A3	-	-	-	25,22	21,91	3,30	-	-	-
75	M65	PTPRF	-	-	-	26,86	21,91	4,95	-	-	-
76	M65	LOC158960	-	-	-	33,85	21,91	11,94	-	-	-
77	M65	PTEN	40,00	26,89	13,11	-	-	-	-	-	-
78	M65	GAPDH	25,62	26,89	-1,27	21,59	21,91	-0,32	27,40	27,39	0,01
79	M65	RPLP0	26,00	26,89	-0,89	20,25	21,91	-1,66	25,89	27,39	-1,50
80	M65	UBC	29,05	26,89	2,16	23,89	21,91	1,98	28,88	27,39	1,50
81	M66	AREG	27,31	23,16	4,16	24,49	21,12	3,37	31,03	28,25	2,77
82	M66	EREG	30,78	23,16	7,62	27,61	21,12	6,49	35,73	28,25	7,48
83	M66	DUSP6	-	-	-	31,84	21,12	10,72	-	-	-
84	M66	SLC26A3	-	-	-	28,31	21,12	7,19	-	-	-
85	M66	PTPRF	-	-	-	26,37	21,12	5,25	-	-	-
86	M66	LOC158960	-	-	-	33,47	21,12	12,35	-	-	-
87	M66	PTEN	34,54	23,16	11,39	-	-	-	-	-	-
88	M66	GAPDH	22,48	23,16	-0,67	20,26	21,12	-0,86	28,28	28,25	0,03
89	M66	RPLP0	20,91	23,16	-2,24	19,75	21,12	-1,37	26,16	28,25	-2,10
90	M66	UBC	26,07	23,16	2,92	23,36	21,12	2,24	30,32	28,25	2,07
91	M60	AREG	30,26	24,15	6,11	-	-	-	31,57	29,82	1,75
92	M60	EREG	31,58	24,15	7,42	-	-	-	35,34	29,82	5,53
93	M60	DUSP6	-	-	-	-	-	-	-	-	-
94	M60	SLC26A3	-	-	-	-	-	-	-	-	-
95	M60	PTPRF	-	-	-	-	-	-	-	-	-
96	M60	LOC158960	-	-	-	-	-	-	-	-	-
97	M60	PTEN	34,37	24,15	10,21	-	-	-	-	-	-
98	M60	GAPDH	23,31	24,15	-0,84	-	-	-	29,11	29,82	-0,71
99	M60	RPLP0	22,78	24,15	-1,38	-	-	-	29,59	29,82	-0,23
100	M60	UBC	26,38	24,15	2,22	-	-	-	30,76	29,82	0,94
101	M68	AREG	27,84	23,10	4,74	26,23	21,66	4,57	33,09	29,26	3,83
102	M68	EREG	30,49	23,10	7,39	27,90	21,66	6,24	35,57	29,26	6,32
103	M68	DUSP6	-	-	-	32,06	21,66	10,40	-	-	-
104	M68	SLC26A3	-	-	-	30,19	21,66	8,53	-	-	-
105	M68	PTPRF	-	-	-	26,03	21,66	4,37	-	-	-
106	M68	LOC158960	-	-	-	33,20	21,66	11,54	-	-	-
107	M68	PTEN	34,15	23,10	11,05	-	-	-	-	-	-

108	M68	GAPDH	22,16	23,10	-0,94	20,62	21,66	-1,04	29,33	29,26	0,07
109	M68	RPLP0	21,98	23,10	-1,12	21,20	21,66	-0,46	28,18	29,26	-1,08
110	M68	UBC	25,17	23,10	2,07	23,16	21,66	1,50	30,26	29,26	1,01
111	M57	AREG	25,17	21,95	3,22	26,26	20,76	5,49	31,56	27,18	4,38
112	M57	EREG	29,19	21,95	7,24	31,28	20,76	10,52	37,60	27,18	10,42
113	M57	DUSP6	-	-	-	31,92	20,76	11,15	-	-	-
114	M57	SLC26A3	-	-	-	21,83	20,76	1,06	-	-	-
115	M57	PTPRF	-	-	-	24,40	20,76	3,64	-	-	-
116	M57	LOC158960	-	-	-	32,28	20,76	11,51	-	-	-
117	M57	PTEN	32,43	21,95	10,48	-	-	-	-	-	-
118	M57	GAPDH	21,02	21,95	-0,93	19,82	20,76	-0,95	27,68	27,18	0,50
119	M57	RPLP0	20,31	21,95	-1,63	20,35	20,76	-0,42	26,68	27,18	-0,50
120	M57	UBC	24,51	21,95	2,56	22,13	20,76	1,36	-	27,18	-
121	M72	AREG	26,76	24,10	2,67	26,45	21,54	4,91	35,22	30,49	4,72
122	M72	EREG	31,14	24,10	7,04	28,83	21,54	7,29	38,20	30,49	7,71
123	M72	DUSP6	-	-	-	33,22	21,54	11,68	-	-	-
124	M72	SLC26A3	-	-	-	28,02	21,54	6,48	-	-	-
125	M72	PTPRF	-	-	-	25,84	21,54	4,31	-	-	-
126	M72	LOC158960	-	-	-	32,70	21,54	11,16	-	-	-
127	M72	PTEN	35,09	24,10	10,99	-	-	-	-	-	-
128	M72	GAPDH	24,05	24,10	-0,05	20,90	21,54	-0,63	31,82	30,49	1,33
129	M72	RPLP0	22,02	24,10	-2,08	21,07	21,54	-0,47	26,78	30,49	-3,71
130	M72	UBC	26,22	24,10	2,12	22,64	21,54	1,11	32,87	30,49	2,38
131	M75	AREG	28,97	22,77	6,20	25,93	20,43	5,49	33,14	29,43	3,71
132	M75	EREG	33,87	22,77	11,11	30,19	20,43	9,75	39,75	29,43	10,32
133	M75	DUSP6	-	-	-	29,85	20,43	9,42	-	-	-
134	M75	SLC26A3	-	-	-	29,53	20,43	9,10	-	-	-
135	M75	PTPRF	-	-	-	23,31	20,43	2,87	-	-	-
136	M75	LOC158960	-	-	-	33,20	20,43	12,77	-	-	-
137	M75	PTEN	32,27	22,77	9,51	-	-	-	-	-	-
138	M75	GAPDH	22,84	22,77	0,08	19,86	20,43	-0,57	30,33	29,43	0,90
139	M75	RPLP0	20,73	22,77	-2,03	19,28	20,43	-1,16	27,07	29,43	-2,36
140	M75	UBC	24,72	22,77	1,96	22,17	20,43	1,73	30,88	29,43	1,46
141	M81	AREG	29,51	22,22	7,29	27,65	19,70	7,95	36,40	30,66	5,75
142	M81	EREG	32,69	22,22	10,47	30,73	19,70	11,03	40,00	30,66	9,34
143	M81	DUSP6	-	-	-	29,55	19,70	9,85	-	-	-
144	M81	SLC26A3	-	-	-	31,70	19,70	11,99	-	-	-
145	M81	PTPRF	-	-	-	25,71	19,70	6,01	-	-	-

146	M81	LOC158960	-	-	-	34,23	19,70	14,53	-	-	-
147	M81	PTEN	33,14	22,22	10,91	-	-	-	-	-	-
148	M81	GAPDH	21,14	22,22	-1,09	18,56	19,70	-1,14	30,22	30,66	-0,44
149	M81	RPLP0	21,04	22,22	-1,19	18,79	19,70	-0,92	27,66	30,66	-3,00
150	M81	UBC	24,49	22,22	2,27	21,76	19,70	2,06	34,09	30,66	3,43
151	M80	AREG	31,25	24,67	6,58	26,53	22,41	4,11	38,30	32,04	6,26
152	M80	EREG	40,00	24,67	15,33	33,20	22,41	10,79	40,00	32,04	7,96
153	M80	DUSP6	-	-	-	33,77	22,41	11,36	-	-	-
154	M80	SLC26A3	-	-	-	30,99	22,41	8,58	-	-	-
155	M80	PTPRF	-	-	-	26,43	22,41	4,02	-	-	-
156	M80	LOC158960	-	-	-	34,72	22,41	12,31	-	-	-
157	M80	PTEN	35,60	24,67	10,93	-	-	-	-	-	-
158	M80	GAPDH	23,38	24,67	-1,28	21,62	22,41	-0,80	32,64	32,04	0,59
159	M80	RPLP0	23,92	24,67	-0,74	21,96	22,41	-0,45	29,41	32,04	-2,64
160	M80	UBC	26,69	24,67	2,02	23,66	22,41	1,24	34,08	32,04	2,04
161	M83	AREG	25,59	22,22	3,36	22,06	20,60	1,46	27,32	26,78	0,54
162	M83	EREG	30,06	22,22	7,84	25,54	20,60	4,94	32,19	26,78	5,41
163	M83	DUSP6	-	-	-	32,00	20,60	11,41	-	-	-
164	M83	SLC26A3	-	-	-	30,11	20,60	9,51	-	-	-
165	M83	PTPRF	-	-	-	25,28	20,60	4,68	-	-	-
166	M83	LOC158960	-	-	-	31,82	20,60	11,23	-	-	-
167	M83	PTEN	32,69	22,22	10,47	-	-	-	-	-	-
168	M83	GAPDH	21,77	22,22	-0,46	20,23	20,60	-0,37	27,21	26,78	0,43
169	M83	RPLP0	20,32	22,22	-1,90	19,36	20,60	-1,24	24,96	26,78	-1,82
170	M83	UBC	24,58	22,22	2,35	22,21	20,60	1,61	28,17	26,78	1,39
171	M88	AREG	26,40	22,95	3,45	22,89	19,84	3,05	28,13	26,09	2,04
172	M88	EREG	29,90	22,95	6,95	26,07	19,84	6,23	30,99	26,09	4,89
173	M88	DUSP6	-	-	-	31,13	19,84	11,29	-	-	-
174	M88	SLC26A3	-	-	-	25,85	19,84	6,01	-	-	-
175	M88	PTPRF	-	-	-	28,55	19,84	8,71	-	-	-
176	M88	LOC158960	-	-	-	32,13	19,84	12,29	-	-	-
177	M88	PTEN	32,14	22,95	9,19	-	-	-	-	-	-
178	M88	GAPDH	22,00	22,95	-0,95	18,77	19,84	-1,07	26,00	26,09	-0,09
179	M88	RPLP0	21,78	22,95	-1,17	19,02	19,84	-0,82	24,60	26,09	-1,49
180	M88	UBC	25,07	22,95	2,12	21,73	19,84	1,89	27,67	26,09	1,58
181	M86	AREG	27,89	21,59	6,30	25,75	21,41	4,34	38,31	34,16	4,14
182	M86	EREG	32,89	21,59	11,29	30,16	21,41	8,74	40,00	34,16	5,84
183	M86	DUSP6	-	-	-	32,24	21,41	10,83	-	-	-

184	M86	SLC26A3	-	-	-	35,18	21,41	13,77	-	-	-
185	M86	PTPRF	-	-	-	26,33	21,41	4,92	-	-	-
186	M86	LOC158960	-	-	-	34,87	21,41	13,46	-	-	-
187	M86	PTEN	32,41	21,59	10,82	-	-	-	-	-	-
188	M86	GAPDH	20,29	21,59	-1,30	20,06	21,41	-1,35	36,89	34,16	2,72
189	M86	RPLP0	19,37	21,59	-2,23	19,92	21,41	-1,49	26,73	34,16	-7,43
190	M86	UBC	25,12	21,59	3,53	24,25	21,41	2,84	38,88	34,16	4,71
191	M89	AREG	28,64	23,45	5,19	23,07	20,63	2,44	28,04	26,86	1,18
192	M89	EREG	31,75	23,45	8,30	27,08	20,63	6,45	32,79	26,86	5,94
193	M89	DUSP6	-	-	-	33,60	20,63	12,98	-	-	-
194	M89	SLC26A3	-	-	-	28,55	20,63	7,93	-	-	-
195	M89	PTPRF	-	-	-	25,21	20,63	4,59	-	-	-
196	M89	LOC158960	-	-	-	34,14	20,63	13,51	-	-	-
197	M89	PTEN	33,71	23,45	10,26	-	-	-	-	-	-
198	M89	GAPDH	23,08	23,45	-0,37	19,63	20,63	-1,00	26,76	26,86	-0,09
199	M89	RPLP0	22,16	23,45	-1,29	19,62	20,63	-1,01	25,70	26,86	-1,15
200	M89	UBC	25,12	23,45	1,67	22,63	20,63	2,01	28,11	26,86	1,25
201	M91	AREG	27,38	22,57	4,80	24,21	20,53	3,68	27,11	25,19	1,93
202	M91	EREG	30,23	22,57	7,66	27,13	20,53	6,60	30,05	25,19	4,86
203	M91	DUSP6	-	-	-	33,26	20,53	12,73	-	-	-
204	M91	SLC26A3	-	-	-	26,41	20,53	5,88	-	-	-
205	M91	PTPRF	-	-	-	24,67	20,53	4,14	-	-	-
206	M91	LOC158960	-	-	-	31,69	20,53	11,16	-	-	-
207	M91	PTEN	32,09	22,57	9,51	-	-	-	-	-	-
208	M91	GAPDH	22,29	22,57	-0,28	19,88	20,53	-0,65	25,21	25,19	0,02
209	M91	RPLP0	21,12	22,57	-1,45	20,10	20,53	-0,43	24,13	25,19	-1,06
210	M91	UBC	24,30	22,57	1,73	21,61	20,53	1,08	26,22	25,19	1,03
211	M92	AREG	28,09	24,39	3,70	23,92	20,47	3,45	29,10	26,35	2,75
212	M92	EREG	30,97	24,39	6,58	26,01	20,47	5,54	31,09	26,35	4,73
213	M92	DUSP6	-	-	-	30,69	20,47	10,22	-	-	-
214	M92	SLC26A3	-	-	-	26,33	20,47	5,86	-	-	-
215	M92	PTPRF	-	-	-	24,42	20,47	3,96	-	-	-
216	M92	LOC158960	-	-	-	32,55	20,47	12,08	-	-	-
217	M92	PTEN	34,99	24,39	10,60	-	-	-	-	-	-
218	M92	GAPDH	24,36	24,39	-0,03	19,88	20,47	-0,59	26,45	26,35	0,09
219	M92	RPLP0	22,98	24,39	-1,41	19,63	20,47	-0,84	25,00	26,35	-1,36
220	M92	UBC	25,83	24,39	1,44	21,90	20,47	1,43	27,62	26,35	1,27
221	M90	AREG	29,14	23,14	5,99	25,26	21,28	3,98	31,22	27,06	4,15

222	M90	EREG	31,75	23,14	8,61	28,42	21,28	7,14	34,15	27,06	7,08
223	M90	DUSP6	-	-	-	31,84	21,28	10,56	-	-	-
224	M90	SLC26A3	-	-	-	29,57	21,28	8,29	-	-	-
225	M90	PTPRF	-	-	-	26,32	21,28	5,03	-	-	-
226	M90	LOC158960	-	-	-	33,22	21,28	11,94	-	-	-
227	M90	PTEN	32,85	23,14	9,71	-	-	-	-	-	-
228	M90	GAPDH	21,80	23,14	-1,35	20,47	21,28	-0,82	26,71	27,06	-0,36
229	M90	RPLP0	21,73	23,14	-1,42	20,19	21,28	-1,09	26,34	27,06	-0,73
230	M90	UBC	25,91	23,14	2,76	23,18	21,28	1,90	28,15	27,06	1,09
231	M13	AREG	26,94	23,26	3,68	21,78	20,17	1,61	30,88	29,56	1,32
232	M13	EREG	30,90	23,26	7,65	26,47	20,17	6,30	36,74	29,56	7,18
233	M13	DUSP6	-	-	-	32,17	20,17	12,00	-	-	-
234	M13	SLC26A3	-	-	-	27,39	20,17	7,22	-	-	-
235	M13	PTPRF	-	-	-	25,96	20,17	5,79	-	-	-
236	M13	LOC158960	-	-	-	33,03	20,17	12,86	-	-	-
237	M13	PTEN	33,92	23,26	10,67	-	-	-	-	-	-
238	M13	GAPDH	22,77	23,26	-0,48	19,54	20,17	-0,63	29,55	29,56	-0,02
239	M13	RPLP0	22,05	23,26	-1,21	18,94	20,17	-1,23	27,41	29,56	-2,15
240	M13	UBC	24,94	23,26	1,69	22,03	20,17	1,86	31,73	29,56	2,17
241	M23	AREG	29,45	24,40	5,05	26,19	21,28	4,91	29,97	26,36	3,61
242	M23	EREG	35,10	24,40	10,70	29,24	21,28	7,96	34,35	26,36	7,99
243	M23	DUSP6	-	-	-	33,44	21,28	12,16	-	-	-
244	M23	SLC26A3	-	-	-	24,82	21,28	3,53	-	-	-
245	M23	PTPRF	-	-	-	25,40	21,28	4,12	-	-	-
246	M23	LOC158960	-	-	-	31,53	21,28	10,25	-	-	-
247	M23	PTEN	33,67	24,40	9,27	-	-	-	-	-	-
248	M23	GAPDH	23,86	24,40	-0,54	19,72	21,28	-1,56	25,48	26,36	-0,88
249	M23	RPLP0	22,26	24,40	-2,14	20,85	21,28	-0,43	25,58	26,36	-0,78
250	M23	UBC	27,08	24,40	2,68	23,28	21,28	2,00	28,02	26,36	1,66
251	M43	AREG	29,01	23,36	5,64	27,94	20,53	7,41	36,82	29,97	6,86
252	M43	EREG	36,44	23,36	13,07	32,55	20,53	12,03	40,00	29,97	10,03
253	M43	DUSP6	-	-	-	30,95	20,53	10,42	-	-	-
254	M43	SLC26A3	-	-	-	30,26	20,53	9,74	-	-	-
255	M43	PTPRF	-	-	-	25,10	20,53	4,57	-	-	-
256	M43	LOC158960	-	-	-	33,59	20,53	13,07	-	-	-
257	M43	PTEN	33,22	23,36	9,86	-	-	-	-	-	-
258	M43	GAPDH	22,41	23,36	-0,96	19,54	20,53	-0,98	29,29	29,97	-0,68
259	M43	RPLP0	23,13	23,36	-0,23	19,91	20,53	-0,62	28,59	29,97	-1,38

260	M43	UBC	24,55	23,36	1,19	22,13	20,53	1,60	32,02	29,97	2,05
261	M53	AREG	30,41	22,85	7,56	28,80	20,60	8,20	40,00	32,73	7,27
262	M53	EREG	32,77	22,85	9,92	31,07	20,60	10,47	40,00	32,73	7,27
263	M53	DUSP6	-	-	-	32,22	20,60	11,62	-	-	-
264	M53	SLC26A3	-	-	-	31,81	20,60	11,21	-	-	-
265	M53	PTPRF	-	-	-	26,45	20,60	5,85	-	-	-
266	M53	LOC158960	-	-	-	33,58	20,60	12,98	-	-	-
267	M53	PTEN	33,96	22,85	11,10	-	-	-	-	-	-
268	M53	GAPDH	21,37	22,85	-1,48	19,62	20,60	-0,98	32,52	32,73	-0,21
269	M53	RPLP0	21,73	22,85	-1,12	20,18	20,60	-0,43	29,92	32,73	-2,81
270	M53	UBC	25,45	22,85	2,60	22,01	20,60	1,41	35,75	32,73	3,02
271	M56	AREG	28,06	23,62	4,45	25,71	23,17	2,54	31,01	28,86	2,15
272	M56	EREG	32,07	23,62	8,45	29,17	23,17	6,00	36,19	28,86	7,33
273	M56	DUSP6	-	-	-	33,05	23,17	9,88	-	-	-
274	M56	SLC26A3	-	-	-	27,01	23,17	3,85	-	-	-
275	M56	PTPRF	-	-	-	26,51	23,17	3,34	-	-	-
276	M56	LOC158960	-	-	-	34,98	23,17	11,82	-	-	-
277	M56	PTEN	34,14	23,62	10,52	-	-	-	-	-	-
278	M56	GAPDH	22,89	23,62	-0,73	22,33	23,17	-0,84	28,36	28,86	-0,50
279	M56	RPLP0	22,29	23,62	-1,33	22,05	23,17	-1,12	28,00	28,86	-0,86
280	M56	UBC	25,68	23,62	2,06	25,13	23,17	1,97	30,21	28,86	1,35
281	M61	AREG	26,18	22,57	3,61	23,57	20,58	2,98	32,47	29,32	3,15
282	M61	EREG	30,02	22,57	7,45	27,44	20,58	6,86	38,00	29,32	8,68
283	M61	DUSP6	-	-	-	31,44	20,58	10,85	-	-	-
284	M61	SLC26A3	-	-	-	28,22	20,58	7,64	-	-	-
285	M61	PTPRF	-	-	-	25,23	20,58	4,64	-	-	-
286	M61	LOC158960	-	-	-	31,61	20,58	11,02	-	-	-
287	M61	PTEN	32,85	22,57	10,28	-	-	-	-	-	-
288	M61	GAPDH	21,88	22,57	-0,69	19,94	20,58	-0,64	28,82	29,32	-0,50
289	M61	RPLP0	21,10	22,57	-1,47	18,86	20,58	-1,73	28,83	29,32	-0,49
290	M61	UBC	24,73	22,57	2,16	22,96	20,58	2,37	30,32	29,32	1,00
291	M29	AREG	25,48	22,82	2,66	23,88	21,05	2,83	29,62	28,03	1,59
292	M29	EREG	30,31	22,82	7,49	28,07	21,05	7,03	35,81	28,03	7,78
293	M29	DUSP6	-	-	-	31,26	21,05	10,21	-	-	-
294	M29	SLC26A3	-	-	-	25,68	21,05	4,64	-	-	-
295	M29	PTPRF	-	-	-	25,95	21,05	4,90	-	-	-
296	M29	LOC158960	-	-	-	33,41	21,05	12,36	-	-	-
297	M29	PTEN	34,88	22,82	12,06	-	-	-	-	-	-

298	M29	GAPDH	22,38	22,82	-0,44	20,46	21,05	-0,59	27,66	28,03	-0,37
299	M29	RPLP0	20,85	22,82	-1,97	20,25	21,05	-0,80	26,74	28,03	-1,29
300	M29	UBC	25,23	22,82	2,41	22,43	21,05	1,38	29,69	28,03	1,66
301	M76	AREG	27,23	25,17	2,06	22,96	19,82	3,14	27,57	26,05	1,51
302	M76	EREG	30,49	25,17	5,32	26,27	19,82	6,45	30,61	26,05	4,56
303	M76	DUSP6	-	-	-	31,61	19,82	11,79	-	-	-
304	M76	SLC26A3	-	-	-	30,20	19,82	10,37	-	-	-
305	M76	PTPRF	-	-	-	24,36	19,82	4,54	-	-	-
306	M76	LOC158960	-	-	-	32,62	19,82	12,80	-	-	-
307	M76	PTEN	35,09	25,17	9,91	-	-	-	-	-	-
308	M76	GAPDH	24,47	25,17	-0,71	18,87	19,82	-0,95	26,01	26,05	-0,05
309	M76	RPLP0	24,15	25,17	-1,03	19,01	19,82	-0,81	25,06	26,05	-1,00
310	M76	UBC	26,91	25,17	1,73	21,59	19,82	1,77	27,10	26,05	1,04
311	M79	AREG	26,57	22,65	3,92	24,83	20,33	4,50	30,16	26,57	3,58
312	M79	EREG	30,50	22,65	7,85	28,67	20,33	8,34	35,14	26,57	8,57
313	M79	DUSP6	-	-	-	33,96	20,33	13,63	-	-	-
314	M79	SLC26A3	-	-	-	27,42	20,33	7,09	-	-	-
315	M79	PTPRF	-	-	-	24,44	20,33	4,11	-	-	-
316	M79	LOC158960	-	-	-	31,32	20,33	10,99	-	-	-
317	M79	PTEN	33,38	22,65	10,73	-	-	-	-	-	-
318	M79	GAPDH	21,85	22,65	-0,80	19,38	20,33	-0,95	26,63	26,57	0,05
319	M79	RPLP0	22,26	22,65	-0,39	20,05	20,33	-0,28	25,65	26,57	-0,92
320	M79	UBC	23,83	22,65	1,19	21,56	20,33	1,23	27,44	26,57	0,87
321	M77	AREG	29,22	23,84	5,38	27,89	21,74	6,15	33,26	28,53	4,73
322	M77	EREG	33,78	23,84	9,94	33,57	21,74	11,83	38,60	28,53	10,07
323	M77	DUSP6	-	-	-	34,05	21,74	12,31	-	-	-
324	M77	SLC26A3	-	-	-	27,76	21,74	6,02	-	-	-
325	M77	PTPRF	-	-	-	27,23	21,74	5,49	-	-	-
326	M77	LOC158960	-	-	-	33,96	21,74	12,22	-	-	-
327	M77	PTEN	33,97	23,84	10,13	-	-	-	-	-	-
328	M77	GAPDH	23,24	23,84	-0,60	21,10	21,74	-0,64	28,47	28,53	-0,06
329	M77	RPLP0	22,69	23,84	-1,15	20,91	21,74	-0,83	27,03	28,53	-1,49
330	M77	UBC	25,60	23,84	1,76	23,20	21,74	1,46	30,08	28,53	1,55
331	M82	AREG	25,49	23,66	1,83	22,32	19,91	2,41	32,34	28,99	3,35
332	M82	EREG	29,27	23,66	5,61	25,33	19,91	5,41	37,09	28,99	8,11
333	M82	DUSP6	-	-	-	30,21	19,91	10,29	-	-	-
334	M82	SLC26A3	-	-	-	25,40	19,91	5,48	-	-	-
335	M82	PTPRF	-	-	-	24,29	19,91	4,37	-	-	-

336	M82	LOC158960	-	-	-	31,03	19,91	11,11	-	-	-
337	M82	PTEN	34,34	23,66	10,68	-	-	-	-	-	-
338	M82	GAPDH	23,52	23,66	-0,14	19,15	19,91	-0,76	28,76	28,99	-0,23
339	M82	RPLP0	21,27	23,66	-2,39	19,15	19,91	-0,76	27,59	28,99	-1,40
340	M82	UBC	26,20	23,66	2,53	21,44	19,91	1,52	30,62	28,99	1,63
341	M84	AREG	26,82	24,32	2,49	23,72	21,73	1,99	38,19	35,30	2,89
342	M84	EREG	29,96	24,32	5,64	27,05	21,73	5,32	40,00	35,30	4,70
343	M84	DUSP6	-	-	-	32,02	21,73	10,29	-	-	-
344	M84	SLC26A3	-	-	-	24,21	21,73	2,48	-	-	-
345	M84	PTPRF	-	-	-	25,10	21,73	3,37	-	-	-
346	M84	LOC158960	-	-	-	32,27	21,73	10,53	-	-	-
347	M84	PTEN	33,41	24,32	9,09	-	-	-	-	-	-
348	M84	GAPDH	22,97	24,32	-1,35	20,27	21,73	-1,46	36,40	35,30	1,10
349	M84	RPLP0	23,18	24,32	-1,14	20,44	21,73	-1,29	31,16	35,30	-4,14
350	M84	UBC	26,82	24,32	2,49	24,48	21,73	2,75	38,33	35,30	3,04
351	M87	AREG	29,70	22,80	6,89	24,49	20,51	3,98	29,64	27,27	2,37
352	M87	EREG	33,58	22,80	10,78	29,74	20,51	9,23	35,09	27,27	7,82
353	M87	DUSP6	-	-	-	31,10	20,51	10,59	-	-	-
354	M87	SLC26A3	-	-	-	26,19	20,51	5,68	-	-	-
355	M87	PTPRF	-	-	-	24,86	20,51	4,35	-	-	-
356	M87	LOC158960	-	-	-	31,91	20,51	11,40	-	-	-
357	M87	PTEN	33,27	22,80	10,47	-	-	-	-	-	-
358	M87	GAPDH	22,03	22,80	-0,78	19,46	20,51	-1,05	26,68	27,27	-0,59
359	M87	RPLP0	21,13	22,80	-1,68	19,54	20,51	-0,97	25,45	27,27	-1,82
360	M87	UBC	25,26	22,80	2,45	22,53	20,51	2,02	29,69	27,27	2,42
361	M53	AREG	30,41	22,85	7,56	28,80	20,60	8,20	40,00	32,73	7,27
362	M53	EREG	32,77	22,85	9,92	31,07	20,60	10,47	40,00	32,73	7,27
363	M53	DUSP6	-	-	-	32,22	20,60	11,62	-	-	-
364	M53	SLC26A3	-	-	-	31,81	20,60	11,21	-	-	-
365	M53	PTPRF	-	-	-	26,45	20,60	5,85	-	-	-
366	M53	LOC158960	-	-	-	33,58	20,60	12,98	-	-	-
367	M53	PTEN	33,96	22,85	11,10	-	-	-	-	-	-
368	M53	GAPDH	21,37	22,85	-1,48	19,62	20,60	-0,98	32,52	32,73	-0,21
369	M53	RPLP0	21,73	22,85	-1,12	20,18	20,60	-0,43	29,92	32,73	-2,81
370	M53	UBC	25,45	22,85	2,60	22,01	20,60	1,41	35,75	32,73	3,02
371	M93	AREG	26,13	23,58	2,55	23,25	21,87	1,38	29,55	28,87	0,68
372	M93	EREG	29,95	23,58	6,36	26,47	21,87	4,60	34,17	28,87	5,29
373	M93	DUSP6	-	-	-	34,33	21,87	12,46	-	-	-

374	M93	SLC26A3	-	-	-	32,62	21,87	10,75	-	-	-
375	M93	PTPRF	-	-	-	25,38	21,87	3,51	-	-	-
376	M93	LOC158960	-	-	-	32,84	21,87	10,97	-	-	-
377	M93	PTEN	40,00	23,58	16,42	-	-	-	-	-	-
378	M93	GAPDH	23,37	23,58	-0,21	21,24	21,87	-0,63	28,23	28,87	-0,64
379	M93	RPLP0	21,74	23,58	-1,84	21,73	21,87	-0,14	28,48	28,87	-0,40
380	M93	UBC	25,64	23,58	2,05	22,64	21,87	0,77	29,91	28,87	1,04
381	M94	AREG	28,87	24,44	4,43	26,33	22,38	3,95	33,23	29,54	3,69
382	M94	EREG	33,01	24,44	8,58	29,75	22,38	7,37	38,50	29,54	8,96
383	M94	DUSP6	-	-	-	34,17	22,38	11,79	-	-	-
384	M94	SLC26A3	-	-	-	30,78	22,38	8,40	-	-	-
385	M94	PTPRF	-	-	-	25,18	22,38	2,80	-	-	-
386	M94	LOC158960	-	-	-	32,87	22,38	10,50	-	-	-
387	M94	PTEN	34,81	24,44	10,37	-	-	-	-	-	-
388	M94	GAPDH	24,21	24,44	-0,23	21,72	22,38	-0,66	29,33	29,54	-0,21
389	M94	RPLP0	22,77	24,44	-1,66	21,26	22,38	-1,12	27,74	29,54	-1,80
390	M94	UBC	26,33	24,44	1,89	24,15	22,38	1,78	31,55	29,54	2,01
391	M95	AREG	27,99	24,35	3,64	24,01	20,86	3,15	38,25	33,98	4,27
392	M95	EREG	32,69	24,35	8,34	28,19	20,86	7,34	40,00	33,98	6,02
393	M95	DUSP6	-	-	-	31,44	20,86	10,58	-	-	-
394	M95	SLC26A3	-	-	-	31,12	20,86	10,26	-	-	-
395	M95	PTPRF	-	-	-	24,93	20,86	4,07	-	-	-
396	M95	LOC158960	-	-	-	31,41	20,86	10,55	-	-	-
397	M95	PTEN	33,40	24,35	9,05	-	-	-	-	-	-
398	M95	GAPDH	24,06	24,35	-0,29	20,16	20,86	-0,70	35,42	33,98	1,44
399	M95	RPLP0	22,98	24,35	-1,37	20,11	20,86	-0,74	27,28	33,98	-6,70
400	M95	UBC	26,02	24,35	1,67	22,30	20,86	1,44	39,23	33,98	5,26
401	M96	AREG	29,34	22,76	6,58	24,68	20,34	4,34	40,00	34,00	6,00
402	M96	EREG	38,56	22,76	15,80	28,97	20,34	8,63	40,00	34,00	6,00
403	M96	DUSP6	-	-	-	31,35	20,34	11,01	-	-	-
404	M96	SLC26A3	-	-	-	34,92	20,34	14,58	-	-	-
405	M96	PTPRF	-	-	-	25,23	20,34	4,89	-	-	-
406	M96	LOC158960	-	-	-	32,78	20,34	12,43	-	-	-
407	M96	PTEN	33,40	22,76	10,64	-	-	-	-	-	-
408	M96	GAPDH	22,00	22,76	-0,76	19,17	20,34	-1,17	36,64	34,00	2,64
409	M96	RPLP0	21,17	22,76	-1,58	19,44	20,34	-0,91	26,58	34,00	-7,42
410	M96	UBC	25,10	22,76	2,34	22,42	20,34	2,08	38,77	34,00	4,77
411	M97	AREG	29,14	23,72	5,42	25,75	20,60	5,15	30,92	28,19	2,73

412	M97	EREG	37,01	23,72	13,30	30,84	20,60	10,24	36,29	28,19	8,11
413	M97	DUSP6	-	-	-	31,61	20,60	11,00	-	-	-
414	M97	SLC26A3	-	-	-	32,53	20,60	11,93	-	-	-
415	M97	PTPRF	-	-	-	25,37	20,60	4,77	-	-	-
416	M97	LOC158960	-	-	-	34,08	20,60	13,48	-	-	-
417	M97	PTEN	34,59	23,72	10,87	-	-	-	-	-	-
418	M97	GAPDH	23,06	23,72	-0,66	19,37	20,60	-1,23	27,28	28,19	-0,91
419	M97	RPLP0	21,87	23,72	-1,85	19,29	20,60	-1,32	26,91	28,19	-1,28
420	M97	UBC	26,22	23,72	2,51	23,15	20,60	2,55	30,38	28,19	2,19
421	M98	AREG	26,73	23,81	2,92	22,60	20,31	2,30	31,16	29,29	1,87
422	M98	EREG	30,28	23,81	6,47	24,96	20,31	4,65	36,12	29,29	6,83
423	M98	DUSP6	-	-	-	32,41	20,31	12,11	-	-	-
424	M98	SLC26A3	-	-	-	27,10	20,31	6,79	-	-	-
425	M98	PTPRF	-	-	-	27,59	20,31	7,28	-	-	-
426	M98	LOC158960	-	-	-	31,08	20,31	10,78	-	-	-
427	M98	PTEN	34,10	23,81	10,29	-	-	-	-	-	-
428	M98	GAPDH	23,75	23,81	-0,06	19,85	20,31	-0,45	29,25	29,29	-0,04
429	M98	RPLP0	21,85	23,81	-1,96	18,59	20,31	-1,71	27,23	29,29	-2,05
430	M98	UBC	25,83	23,81	2,02	22,48	20,31	2,17	31,38	29,29	2,09
431	M99	AREG	25,69	23,34	2,35	21,53	19,49	2,04	30,74	27,81	2,93
432	M99	EREG	29,51	23,34	6,18	25,33	19,49	5,84	35,10	27,81	7,30
433	M99	DUSP6	-	-	-	32,02	19,49	12,53	-	-	-
434	M99	SLC26A3	-	-	-	20,92	19,49	1,43	-	-	-
435	M99	PTPRF	-	-	-	23,92	19,49	4,43	-	-	-
436	M99	LOC158960	-	-	-	31,86	19,49	12,37	-	-	-
437	M99	PTEN	33,92	23,34	10,58	-	-	-	-	-	-
438	M99	GAPDH	23,31	23,34	-0,03	18,71	19,49	-0,77	27,77	27,81	-0,03
439	M99	RPLP0	21,85	23,34	-1,49	18,60	19,49	-0,88	26,46	27,81	-1,35
440	M99	UBC	24,86	23,34	1,52	21,14	19,49	1,66	29,18	27,81	1,38
441	M101	AREG	27,69	23,06	4,63	24,85	21,41	3,44	40,00	32,71	7,29
442	M101	EREG	30,78	23,06	7,72	27,14	21,41	5,73	40,00	32,71	7,29
443	M101	DUSP6	-	-	-	31,57	21,41	10,16	-	-	-
444	M101	SLC26A3	-	-	-	30,19	21,41	8,78	-	-	-
445	M101	PTPRF	-	-	-	26,31	21,41	4,90	-	-	-
446	M101	LOC158960	-	-	-	33,56	21,41	12,15	-	-	-
447	M101	PTEN	33,38	23,06	10,32	-	-	-	-	-	-
448	M101	GAPDH	22,47	23,06	-0,59	21,05	21,41	-0,35	38,27	32,71	5,56
449	M101	RPLP0	21,72	23,06	-1,34	20,26	21,41	-1,15	27,16	32,71	-5,56

450	M101	UBC	24,99	23,06	1,93	22,91	21,41	1,50	-	32,71	-
451	M102	AREG	28,52	22,75	5,77	24,50	20,29	4,21	32,31	28,39	3,93
452	M102	EREG	32,36	22,75	9,61	28,96	20,29	8,67	36,50	28,39	8,11
453	M102	DUSP6	-	-	-	30,92	20,29	10,63	-	-	-
454	M102	SLC26A3	-	-	-	27,57	20,29	7,28	-	-	-
455	M102	PTPRF	-	-	-	26,21	20,29	5,92	-	-	-
456	M102	LOC158960	-	-	-	32,58	20,29	12,30	-	-	-
457	M102	PTEN	34,53	22,75	11,78	-	-	-	-	-	-
458	M102	GAPDH	22,25	22,75	-0,50	19,57	20,29	-0,72	28,00	28,39	-0,39
459	M102	RPLP0	21,20	22,75	-1,54	18,47	20,29	-1,82	27,11	28,39	-1,27
460	M102	UBC	24,79	22,75	2,04	22,83	20,29	2,54	30,05	28,39	1,66
461	M104	AREG	27,13	22,77	4,36	26,50	21,33	5,17	32,22	27,99	4,23
462	M104	EREG	31,08	22,77	8,31	28,97	21,33	7,65	34,89	27,99	6,90
463	M104	DUSP6	-	-	-	32,21	21,33	10,89	-	-	-
464	M104	SLC26A3	-	-	-	29,08	21,33	7,76	-	-	-
465	M104	PTPRF	-	-	-	25,13	21,33	3,80	-	-	-
466	M104	LOC158960	-	-	-	33,58	21,33	12,25	-	-	-
467	M104	PTEN	33,99	22,77	11,22	-	-	-	-	-	-
468	M104	GAPDH	21,74	22,77	-1,04	20,52	21,33	-0,81	27,73	27,99	-0,25
469	M104	RPLP0	21,23	22,77	-1,54	20,23	21,33	-1,10	26,63	27,99	-1,35
470	M104	UBC	25,35	22,77	2,58	23,23	21,33	1,91	29,60	27,99	1,61
471	M105	AREG	33,66	22,81	10,86	27,70	22,64	5,06	33,17	28,33	4,84
472	M105	EREG	39,02	22,81	16,22	33,28	22,64	10,64	39,22	28,33	10,89
473	M105	DUSP6	-	-	-	33,94	22,64	11,30	-	-	-
474	M105	SLC26A3	-	-	-	30,07	22,64	7,43	-	-	-
475	M105	PTPRF	-	-	-	28,18	22,64	5,55	-	-	-
476	M105	LOC158960	-	-	-	35,00	22,64	12,36	-	-	-
477	M105	PTEN	34,00	22,81	11,20	-	-	-	-	-	-
478	M105	GAPDH	21,94	22,81	-0,87	21,70	22,64	-0,93	28,20	28,33	-0,13
479	M105	RPLP0	20,67	22,81	-2,14	21,29	22,64	-1,35	26,82	28,33	-1,51
480	M105	UBC	25,81	22,81	3,00	24,92	22,64	2,28	29,97	28,33	1,64
481	M107	AREG	28,64	24,16	4,49	21,86	19,37	2,48	31,16	28,87	2,29
482	M107	EREG	32,51	24,16	8,36	24,06	19,37	4,68	35,19	28,87	6,33
483	M107	DUSP6	-	-	-	28,42	19,37	9,05	-	-	-
484	M107	SLC26A3	-	-	-	26,87	19,37	7,50	-	-	-
485	M107	PTPRF	-	-	-	23,29	19,37	3,92	-	-	-
486	M107	LOC158960	-	-	-	30,23	19,37	10,85	-	-	-
487	M107	PTEN	36,17	24,16	12,01	-	-	-	-	-	-

488	M107	GAPDH	24,16	24,16	0,00	18,49	19,37	-0,88	29,42	28,87	0,56
489	M107	RPLP0	23,06	24,16	-1,09	19,98	19,37	0,60	27,05	28,87	-1,81
490	M107	UBC	25,25	24,16	1,09	19,65	19,37	0,28	30,12	28,87	1,26
491	M106	AREG	25,81	22,74	3,07	22,11	19,16	2,95	28,71	27,17	1,54
492	M106	EREG	30,29	22,74	7,55	26,20	19,16	7,04	34,11	27,17	6,94
493	M106	DUSP6	-	-	-	30,93	19,16	11,77	-	-	-
494	M106	SLC26A3	-	-	-	27,33	19,16	8,17	-	-	-
495	M106	PTPRF	-	-	-	24,29	19,16	5,13	-	-	-
496	M106	LOC158960	-	-	-	31,14	19,16	11,98	-	-	-
497	M106	PTEN	33,21	22,74	10,47	-	-	-	-	-	-
498	M106	GAPDH	21,40	22,74	-1,34	17,96	19,16	-1,20	26,60	27,17	-0,57
499	M106	RPLP0	22,44	22,74	-0,30	18,46	19,16	-0,70	25,85	27,17	-1,32
500	M106	UBC	24,38	22,74	1,64	21,06	19,16	1,90	29,07	27,17	1,89
501	M110	AREG	29,90	23,20	6,70	27,19	20,75	6,44	36,34	30,04	6,30
502	M110	EREG	32,62	23,20	9,42	32,29	20,75	11,54	40,00	30,04	9,96
503	M110	DUSP6	-	-	-	31,60	20,75	10,85	-	-	-
504	M110	SLC26A3	-	-	-	28,74	20,75	7,99	-	-	-
505	M110	PTPRF	-	-	-	24,00	20,75	3,26	-	-	-
506	M110	LOC158960	-	-	-	32,78	20,75	12,03	-	-	-
507	M110	PTEN	33,74	23,20	10,54	-	-	-	-	-	-
508	M110	GAPDH	22,31	23,20	-0,89	19,86	20,75	-0,89	30,25	30,04	0,21
509	M110	RPLP0	21,11	23,20	-2,09	19,25	20,75	-1,50	26,57	30,04	-3,47
510	M110	UBC	26,18	23,20	2,98	23,13	20,75	2,38	33,30	30,04	3,26
511	M112	AREG	27,45	23,59	3,86	27,34	24,22	3,12	29,69	27,86	1,83
512	M112	EREG	31,76	23,59	8,17	32,73	24,22	8,51	34,28	27,86	6,42
513	M112	DUSP6	-	-	-	35,44	24,22	11,23	-	-	-
514	M112	SLC26A3	-	-	-	29,26	24,22	5,04	-	-	-
515	M112	PTPRF	-	-	-	27,16	24,22	2,95	-	-	-
516	M112	LOC158960	-	-	-	37,41	24,22	13,19	-	-	-
517	M112	PTEN	35,28	23,59	11,69	-	-	-	-	-	-
518	M112	GAPDH	23,34	23,59	-0,25	24,01	24,22	-0,21	27,71	27,86	-0,15
519	M112	RPLP0	21,59	23,59	-2,01	23,15	24,22	-1,06	26,76	27,86	-1,10
520	M112	UBC	25,86	23,59	2,26	25,49	24,22	1,27	29,12	27,86	1,26
521	M115	AREG	30,75	24,25	6,50	25,75	20,38	5,36	31,23	27,85	3,38
522	M115	EREG	33,24	24,25	9,00	27,64	20,38	7,26	35,30	27,85	7,44
523	M115	DUSP6	-	-	-	32,38	20,38	11,99	-	-	-
524	M115	SLC26A3	-	-	-	31,93	20,38	11,55	-	-	-
525	M115	PTPRF	-	-	-	24,93	20,38	4,54	-	-	-

526	M115	LOC158960	-	-	-	32,34	20,38	11,95	-	-	-
527	M115	PTEN	34,04	24,25	9,79	-	-	-	-	-	-
528	M115	GAPDH	23,49	24,25	-0,76	19,53	20,38	-0,86	27,89	27,85	0,04
529	M115	RPLP0	22,74	24,25	-1,51	19,22	20,38	-1,16	26,75	27,85	-1,11
530	M115	UBC	26,51	24,25	2,27	22,40	20,38	2,02	28,92	27,85	1,07
531	M117	AREG	30,13	23,66	6,47	28,36	21,70	6,66	30,66	25,64	5,03
532	M117	EREG	32,61	23,66	8,94	31,69	21,70	9,99	33,47	25,64	7,83
533	M117	DUSP6	-	-	-	33,08	21,70	11,38	-	-	-
534	M117	SLC26A3	-	-	-	25,27	21,70	3,56	-	-	-
535	M117	PTPRF	-	-	-	25,61	21,70	3,91	-	-	-
536	M117	LOC158960	-	-	-	32,79	21,70	11,08	-	-	-
537	M117	PTEN	32,53	23,66	8,86	-	-	-	-	-	-
538	M117	GAPDH	23,50	23,66	-0,16	21,40	21,70	-0,31	25,81	25,64	0,17
539	M117	RPLP0	22,29	23,66	-1,37	21,02	21,70	-0,68	25,01	25,64	-0,63
540	M117	UBC	25,20	23,66	1,53	22,69	21,70	0,99	26,09	25,64	0,45
541	M118	AREG	29,82	24,70	5,12	24,31	20,42	3,90	31,32	28,51	2,81
542	M118	EREG	33,03	24,70	8,33	27,30	20,42	6,88	35,63	28,51	7,12
543	M118	DUSP6	-	-	-	31,18	20,42	10,76	-	-	-
544	M118	SLC26A3	-	-	-	27,35	20,42	6,93	-	-	-
545	M118	PTPRF	-	-	-	25,28	20,42	4,86	-	-	-
546	M118	LOC158960	-	-	-	33,13	20,42	12,71	-	-	-
547	M118	PTEN	33,96	24,70	9,26	-	-	-	-	-	-
548	M118	GAPDH	24,42	24,70	-0,28	19,21	20,42	-1,21	28,67	28,51	0,16
549	M118	RPLP0	23,92	24,70	-0,78	19,81	20,42	-0,61	27,26	28,51	-1,25
550	M118	UBC	25,76	24,70	1,06	22,23	20,42	1,82	29,59	28,51	1,08
551	M114	AREG	28,79	24,41	4,38	22,42	20,81	1,60	30,13	27,77	2,36
552	M114	EREG	32,56	24,41	8,15	26,32	20,81	5,51	34,51	27,77	6,74
553	M114	DUSP6	-	-	-	30,77	20,81	9,95	-	-	-
554	M114	SLC26A3	-	-	-	23,29	20,81	2,47	-	-	-
555	M114	PTPRF	-	-	-	24,84	20,81	4,02	-	-	-
556	M114	LOC158960	-	-	-	33,68	20,81	12,86	-	-	-
557	M114	PTEN	34,30	24,41	9,89	-	-	-	-	-	-
558	M114	GAPDH	24,03	24,41	-0,37	19,86	20,81	-0,95	27,81	27,77	0,04
559	M114	RPLP0	23,69	24,41	-0,72	20,34	20,81	-0,47	27,10	27,77	-0,67
560	M114	UBC	25,50	24,41	1,09	22,24	20,81	1,43	28,41	27,77	0,64
561	M122	AREG	26,09	23,90	2,19	20,57	18,44	2,13	29,96	28,92	1,04
562	M122	EREG	29,87	23,90	5,98	25,07	18,44	6,63	35,96	28,92	7,04
563	M122	DUSP6	-	-	-	30,32	18,44	11,88	-	-	-

564	M122	SLC26A3	-	-	-	23,25	18,44	4,81	-	-	-
565	M122	PTPRF	-	-	-	30,47	18,44	12,03	-	-	-
566	M122	LOC158960	-	-	-	31,75	18,44	13,31	-	-	-
567	M122	PTEN	33,48	23,90	9,59	-	-	-	-	-	-
568	M122	GAPDH	23,84	23,90	-0,06	17,60	18,44	-0,84	28,45	28,92	-0,47
569	M122	RPLP0	22,02	23,90	-1,87	17,66	18,44	-0,78	27,42	28,92	-1,50
570	M122	UBC	25,83	23,90	1,93	20,06	18,44	1,62	30,89	28,92	1,97
571	M123	AREG	28,25	22,63	5,63	25,45	20,66	4,79	33,15	26,87	6,28
572	M123	EREG	30,23	22,63	7,61	26,48	20,66	5,81	35,33	26,87	8,46
573	M123	DUSP6	-	-	-	29,85	20,66	9,19	-	-	-
574	M123	SLC26A3	-	-	-	38,41	20,66	17,75	-	-	-
575	M123	PTPRF	-	-	-	25,16	20,66	4,50	-	-	-
576	M123	LOC158960	-	-	-	32,22	20,66	11,55	-	-	-
577	M123	PTEN	32,51	22,63	9,88	-	-	-	-	-	-
578	M123	GAPDH	21,89	22,63	-0,73	19,16	20,66	-1,51	26,50	26,87	-0,38
579	M123	RPLP0	20,94	22,63	-1,69	20,00	20,66	-0,67	25,17	26,87	-1,71
580	M123	UBC	25,05	22,63	2,42	22,84	20,66	2,17	28,96	26,87	2,09
581	M124	AREG	29,96	24,70	5,25	23,66	20,71	2,95	31,79	29,14	2,65
582	M124	EREG	30,92	24,70	6,21	26,54	20,71	5,83	36,95	29,14	7,81
583	M124	DUSP6	-	-	-	32,29	20,71	11,58	-	-	-
584	M124	SLC26A3	-	-	-	23,86	20,71	3,15	-	-	-
585	M124	PTPRF	-	-	-	25,08	20,71	4,37	-	-	-
586	M124	LOC158960	-	-	-	32,11	20,71	11,40	-	-	-
587	M124	PTEN	33,63	24,70	8,93	-	-	-	-	-	-
588	M124	GAPDH	24,66	24,70	-0,04	20,24	20,71	-0,47	29,77	29,14	0,63
589	M124	RPLP0	23,58	24,70	-1,12	19,75	20,71	-0,96	27,13	29,14	-2,01
590	M124	UBC	25,86	24,70	1,16	22,13	20,71	1,43	30,51	29,14	1,37
591	M120	AREG	28,41	22,93	5,48	27,30	21,31	5,99	31,57	26,40	5,17
592	M120	EREG	39,04	22,93	16,12	33,95	21,31	12,64	36,06	26,40	9,67
593	M120	DUSP6	-	-	-	32,68	21,31	11,37	-	-	-
594	M120	SLC26A3	-	-	-	37,43	21,31	16,12	-	-	-
595	M120	PTPRF	-	-	-	25,74	21,31	4,44	-	-	-
596	M120	LOC158960	-	-	-	34,28	21,31	12,98	-	-	-
597	M120	PTEN	32,52	22,93	9,60	-	-	-	-	-	-
598	M120	GAPDH	22,13	22,93	-0,79	20,76	21,31	-0,55	25,83	26,40	-0,56
599	M120	RPLP0	21,77	22,93	-1,16	20,02	21,31	-1,29	26,28	26,40	-0,12
600	M120	UBC	24,88	22,93	1,95	23,15	21,31	1,84	27,08	26,40	0,68
601	M125	AREG	30,22	22,52	7,70	23,61	21,17	2,43	29,00	27,40	1,60

602	M125	EREG	37,85	22,52	15,32	28,44	21,17	7,27	34,32	27,40	6,92
603	M125	DUSP6	-	-	-	33,15	21,17	11,98	-	-	-
604	M125	SLC26A3	-	-	-	23,71	21,17	2,54	-	-	-
605	M125	PTPRF	-	-	-	26,38	21,17	5,21	-	-	-
606	M125	LOC158960	-	-	-	32,86	21,17	11,69	-	-	-
607	M125	PTEN	32,83	22,52	10,31	-	-	-	-	-	-
608	M125	GAPDH	22,52	22,52	0,00	20,56	21,17	-0,62	27,10	27,40	-0,30
609	M125	RPLP0	20,48	22,52	-2,05	19,51	21,17	-1,66	26,10	27,40	-1,31
610	M125	UBC	24,58	22,52	2,05	23,45	21,17	2,28	29,01	27,40	1,61
611	M128	AREG	26,16	23,49	2,67	24,96	20,38	4,58	35,10	32,86	2,24
612	M128	EREG	32,02	23,49	8,53	28,83	20,38	8,45	40,00	32,86	7,14
613	M128	DUSP6	-	-	-	30,43	20,38	10,05	-	-	-
614	M128	SLC26A3	-	-	-	25,44	20,38	5,06	-	-	-
615	M128	PTPRF	-	-	-	24,22	20,38	3,84	-	-	-
616	M128	LOC158960	-	-	-	33,21	20,38	12,83	-	-	-
617	M128	PTEN	36,23	23,49	12,74	-	-	-	-	-	-
618	M128	GAPDH	23,04	23,49	-0,45	19,37	20,38	-1,01	32,63	32,86	-0,23
619	M128	RPLP0	21,26	23,49	-2,23	19,65	20,38	-0,72	30,85	32,86	-2,01
620	M128	UBC	26,17	23,49	2,68	22,11	20,38	1,73	35,09	32,86	2,24
621	M129	AREG	27,79	24,21	3,58	24,94	20,89	4,05	30,85	26,82	4,03
622	M129	EREG	32,40	24,21	8,18	28,20	20,89	7,31	34,36	26,82	7,54
623	M129	DUSP6	-	-	-	29,50	20,89	8,61	-	-	-
624	M129	SLC26A3	-	-	-	31,48	20,89	10,58	-	-	-
625	M129	PTPRF	-	-	-	24,94	20,89	4,04	-	-	-
626	M129	LOC158960	-	-	-	31,43	20,89	10,54	-	-	-
627	M129	PTEN	34,58	24,21	10,37	-	-	-	-	-	-
628	M129	GAPDH	24,48	24,21	0,27	20,54	20,89	-0,35	26,79	26,82	-0,03
629	M129	RPLP0	22,70	24,21	-1,51	20,00	20,89	-0,89	25,58	26,82	-1,25
630	M129	UBC	25,46	24,21	1,24	22,14	20,89	1,24	28,10	26,82	1,28
631	M132	AREG	31,39	23,72	7,67	27,31	20,80	6,51	33,30	29,25	4,05
632	M132	EREG	35,06	23,72	11,34	30,88	20,80	10,09	37,85	29,25	8,61
633	M132	DUSP6	-	-	-	31,41	20,80	10,61	-	-	-
634	M132	SLC26A3	-	-	-	30,91	20,80	10,11	-	-	-
635	M132	PTPRF	-	-	-	25,31	20,80	4,51	-	-	-
636	M132	LOC158960	-	-	-	33,40	20,80	12,60	-	-	-
637	M132	PTEN	33,70	23,72	9,98	-	-	-	-	-	-
638	M132	GAPDH	23,07	23,72	-0,65	19,87	20,80	-0,93	28,56	29,25	-0,69
639	M132	RPLP0	21,91	23,72	-1,81	19,78	20,80	-1,02	27,45	29,25	-1,80

640	M132	UBC	26,18	23,72	2,46	22,75	20,80	1,95	31,74	29,25	2,49
641	M121	AREG	29,35	23,37	5,98	28,21	20,43	7,78	36,50	30,30	6,20
642	M121	EREG	40,00	23,37	16,63	37,40	20,43	16,97	40,00	30,30	9,70
643	M121	DUSP6	-	-	-	30,98	20,43	10,55	-	-	-
644	M121	SLC26A3	-	-	-	36,66	20,43	16,23	-	-	-
645	M121	PTPRF	-	-	-	26,44	20,43	6,01	-	-	-
646	M121	LOC158960	-	-	-	32,88	20,43	12,45	-	-	-
647	M121	PTEN	34,15	23,37	10,78	-	-	-	-	-	-
648	M121	GAPDH	22,51	23,37	-0,86	19,83	20,43	-0,60	30,22	30,30	-0,09
649	M121	RPLP0	21,84	23,37	-1,53	19,66	20,43	-0,77	29,13	30,30	-1,17
650	M121	UBC	25,76	23,37	2,39	21,80	20,43	1,37	31,56	30,30	1,26
651	M33	AREG	30,7	22,65	8,01	28,22	22,6	5,6	-	-	-
652	M33	EREG	36,2	22,65	13,51	33,86	22,6	11,3	-	-	-
653	M33	DUSP6	-	-	-	32,856	22,6	10,3	-	-	-
654	M33	SLC26A3	-	-	-	35,523	22,6	12,9	-	-	-
655	M33	PTPRF	-	-	-	26,778	22,6	4,2	-	-	-
656	M33	LOC158960	-	-	-	34,299	22,6	11,7	-	-	-
657	M33	PTEN	33,8	22,65	11,17	-	-	-	-	-	-
658	M33	GAPDH	21,8	22,65	-0,89	22,135	22,6	-0,4	-	-	-
659	M33	RPLP0	21,5	22,65	-1,13	21,036	22,6	-1,5	-	-	-
660	M33	UBC	24,7	22,65	2,02	24,571	22,6	2,0	-	-	-
661	M47	AREG	28,3	22,78	5,56	29,241	24,3	4,9	-	-	-
662	M47	EREG	31,6	22,78	8,82	33,138	24,3	8,8	-	-	-
663	M47	DUSP6	-	-	-	34,099	24,3	9,8	-	-	-
664	M47	SLC26A3	-	-	-	28,36	24,3	4,0	-	-	-
665	M47	PTPRF	-	-	-	29,229	24,3	4,9	-	-	-
666	M47	LOC158960	-	-	-	36,258	24,3	11,9	-	-	-
667	M47	PTEN	32,9	22,78	10,09	-	-	-	-	-	-
668	M47	GAPDH	21,9	22,78	-0,92	23,75	24,3	-0,6	-	-	-
669	M47	RPLP0	21,7	22,78	-1,03	24,007	24,3	-0,3	-	-	-
670	M47	UBC	24,7	22,78	1,95	25,255	24,3	0,9	-	-	-
671	M85	AREG	31,3	25,50	5,81	27,619	20,3	7,4	-	-	-
672	M85	EREG	39,2	25,50	13,70	33,722	20,3	13,5	-	-	-
673	M85	DUSP6	-	-	-	30,819	20,3	10,6	-	-	-
674	M85	SLC26A3	-	-	-	34,428	20,3	14,2	-	-	-
675	M85	PTPRF	-	-	-	24,343	20,3	4,1	-	-	-
676	M85	LOC158960	-	-	-	32,3	20,3	12,0	-	-	-
677	M85	PTEN	34,1	25,50	8,59	-	-	-	-	-	-

678	M85	GAPDH	24,7	25,50	-0,79	18,935	20,3	-1,3	-	-	-
679	M85	RPLP0	24,5	25,50	-0,99	19,546	20,3	-0,7	-	-	-
680	M85	UBC	27,3	25,50	1,77	22,284	20,3	2,0	-	-	-

Tab.S9. *Cetuximab responders (R) and non-responders (NR) sorted according to the normalized mean of the Ct expression values of the AREG and EREG (Cut-off Value was set at 6); The responders are characterized with lower Ct mean of AREG and EREG (higher expression of the two genes), while non-responders have higher CT mean of AREG and EREG (lower expression levels); AREG and EREG were measured directly in the xenografts that served as controls in the treatment experiment, green - NR, incorrectly classified ba AREG/EREG, but mutated in one of the 3 genes: KRAS; BRAF; PIK3CA; blue - NR, correctly classified ba AREG/EREG, and WT in all of the 3 genes: KRAS; BRAF; PIK3CA; orange - R, incorrectly classified by AREG/EREG and WT all of the 3 genes: KRAS; BRAF; PIK3CA*

No	model-ID	AREG Norm	EREG Norm	AREG + EREG Norm	CE Resp.	T/C		KRAS	BRAF	PIK3CA	Mut No
1	M76	2,1	5,3	3,7	R	1,73	++++	WT	WT	WT	0
2	M82	1,8	5,6	3,7	R	3,64	++++	WT	WT	WT	0
3	M84	2,5	5,6	4,1	R	17,24	+++	WT	WT	WT	0
4	M122	2,2	6,0	4,1	R	3,19	++++	WT	WT	WT	0
5	M99	2,4	6,2	4,3	R	13,12	+++	WT	WT	WT	0
6	M93	2,5	6,4	4,5	R	7	+++	WT	WT	WT	0
7	M98	2,9	6,5	4,7	R	8,56	+++	WT	WT	WT	0
8	M72	2,7	7,0	4,9	NR	38,1	+	WT	WT	WT	0
9	M29	2,7	7,5	5,1	R	8,9	+++	WT	WT	WT	0
10	M92	3,7	6,6	5,1	NR	74,65	-	35 G>T	WT	WT	1
11	M88	3,5	6,9	5,2	R	2,7	++++	WT	WT	WT	0
12	M57	3,2	7,2	5,2	R	19,6	+++	38 G>A	WT	WT	1
13	M106	3,1	7,5	5,3	NR	53,15	-	35 G>T	WT	WT	1
14	M55	3,9	6,8	5,4	NR	58,3	-	WT	WT	WT	0
15	M61	3,6	7,5	5,5	NR	61	-	35 G>T	WT	WT	1
16	M128	2,7	8,5	5,6	R	1,19	++++	34 G>A	WT	WT	1
17	M83	3,4	7,8	5,6	NR	32	++	35 G>C	WT	WT	1
18	M13	3,7	7,6	5,7	R	7,6	+++	WT	WT	WT	0
19	M124	5,3	6,2	5,7	R	17,54	+++	WT	WT	WT	0
20	M129	3,6	8,2	5,9	NR	47,71	+	35 G>T	WT	WT	1
21	M79	3,9	7,9	5,9	R	12	+++	WT	WT	WT	0
22	M66	4,2	7,6	5,9	NR	48,6	+	183 A>T	WT	WT	1
23	M95	3,6	8,3	6,0	R	17,31	+++	38 G>A	WT	WT	1

24	M112	3,9	8,2	6,0	R	5,26	+++	WT	WT	WT	0
25	M68	4,7	7,4	6,1	NR	55,2	-	35 G>T	WT	WT	1
26	M101	4,6	7,7	6,2	NR	65,71	-	34 G>A, T	WT	WT	1
27	M91	4,8	7,7	6,2	NR	35,3	++	WT	WT	WT	0
28	M114	4,4	8,1	6,3	NR	27,08	++	35 G>C	WT	WT	1
29	M104	4,4	8,3	6,3	NR	51,61	-	38 G>A	WT	WT	1
30	M107	4,5	8,4	6,4	NR	42,86	+	38 G>A	WT	WT	1
31	M56	4,4	8,4	6,4	NR	88	-	35 G>T	WT	WT	1
32	M94	4,4	8,6	6,5	NR	46,67	+	WT	WT	WT	0
33	M52	4,4	8,9	6,6	NR	24,8	++	35 G>C	WT	WT	1
34	M123	5,6	7,6	6,6	NR	63,4	-	WT	1799 T>A	WT	1
35	M1	4,2	9,2	6,7	R	4,5	++++	WT	WT	WT	0
36	M118	5,1	8,3	6,7	NR	51,16	-	35 G>T	WT	3140 A>G	2
37	M89	5,2	8,3	6,7	NR	62,5	-	WT	WT	WT	0
38	M60	6,1	7,4	6,8	R	5,26	+++	WT	WT	WT	0
39	M27	4,0	9,6	6,8	NR	36,9	+	35 G>A	WT	WT	1
40	M47	5,6	8,8	7,2	NR	22	++	35 G>C	WT	WT	1
41	M90	6,0	8,6	7,3	NR	79,3	-	35 G>T	WT	1633 G>A	2
42	M77	5,4	9,9	7,7	NR	64,41	-	WT	WT	WT	0
43	M102	5,8	9,6	7,7	NR	27,97	++	35 G>T	WT	WT	1
44	M117	6,5	8,9	7,7	NR	33,78	++	WT	WT	1633 G>A	1
45	M115	6,5	9,0	7,7	NR	82,14	-	38 G>A	WT	WT	1
46	M23	5,0	10,7	7,9	NR	43,7	+	WT	WT	WT	0
47	M110	6,7	9,4	8,1	NR	79,17	-	182 A>T	WT	1624 G>A	2
48	M75	6,2	11,1	8,7	NR	39,6	+	35 G>C	WT	WT	1
49	M65	6,5	11,0	8,7	NR	81,4	-	436 G>A	WT	1633 G>A	2
50	M53	7,6	9,9	8,7	NR	40,8	+	35 G>A	WT	WT	1
51	M86	6,3	11,3	8,8	NR	62,5	-	35 G>A	WT	WT	1
52	M87	6,9	10,8	8,8	NR	45,6	+	WT	WT	WT	0
53	M81	7,3	10,5	8,9	NR	60,87	-	WT	1799 T>A	WT	1
54	M18	7,0	11,0	9,0	NR	67,8	-	35 G>A	WT	WT	1
55	M43	5,6	13,1	9,4	NR	72,7	-	WT	1799 T>A	WT	1
56	M97	5,4	13,3	9,4	NR	89,69	-	WT	WT	WT	0
57	M132	7,7	11,3	9,5	NR	49,23	+	436 G>A	WT	3140 A>G	2

58	M85	5,8	13,7	9,8	NR	27	++	35 G>A	WT	WT	1
59	M59	7,3	13,1	10,2	NR	81	-	WT	1799 T>A	WT	1
60	M63	8,3	12,5	10,4	NR	79,2	-	38 G>A	WT	WT	1
61	M33	8,0	13,5	10,8	NR	94,7	-	WT	1799 T>A	WT	1
62	M120	5,5	16,1	10,8	NR	69,7	-	WT	1799 T>A	WT	1
63	M80	6,6	15,3	11,0	NR	42,4	+	WT	WT	WT	0
64	M96	6,6	15,8	11,2	NR	120	-	35 G>T	WT	1633 G>A	2
65	M121	6,0	16,6	11,3	NR	69,32	-	WT	1799 T>A	WT	1
66	M125	7,7	15,3	11,5	NR	93	-	35 G>T	WT	WT	1
67	M105	10,9	16,2	13,5	NR	73,1	-	WT	WT	WT	0

Tab.S10. A) *Performance of the mRNA signature in terms of sensitivity and specificity during discovery on the xenografts used in the treatment experiment, sorted according to the number of probesets included in the signature; ; mRNA expression analysis performed on the Affymetrix U133 Plus 2.0 GeneChip*

Genes No included in the signature	Sensitivity	Specificity
genes.1.to.3	76,56%	74,89%
genes.1.to.4	76,78%	76,24%
genes.1.to.5	77,33%	77,30%
genes.1.to.6	77,44%	77,95%
genes.1.to.7	78,78%	78,90%
genes.1.to.10	80,56%	80,25%
genes.1.to.11	80,67%	80,38%
genes.1.to.12	80,33%	80,74%
genes.1.to.13	80,44%	80,99%
genes.1.to.14	81,22%	81,16%
genes.1.to.20	82,44%	81,28%
genes.1.to.21	83,44%	81,11%
genes.1.to.22	83,44%	81,02%
genes.1.to.23	83,22%	81,36%
genes.1.to.24	84,22%	81,06%
genes.1.to.30	83,44%	81,44%
genes.1.to.31	83,11%	81,35%
genes.1.to.32	83,67%	81,52%
genes.1.to.33	84,33%	81,47%
genes.1.to.34	84,00%	81,57%
genes.1.to.40	84,00%	81,43%
genes.1.to.41	84,11%	81,55%
genes.1.to.42	84,44%	81,52%
genes.1.to.43	83,78%	81,37%
genes.1.to.44	83,44%	81,30%
genes.1.to.50	84,22%	81,08%
genes.1.to.51	84,78%	80,96%
genes.1.to.52	84,78%	80,91%
genes.1.to.53	84,89%	81,05%
genes.1.to.54	85,22%	81,08%
genes.1.to.100	84,56%	80,05%
genes.1.to.101	84,56%	80,04%
genes.1.to.102	85,00%	79,97%
genes.1.to.103	84,89%	79,98%
genes.1.to.104	84,67%	79,96%
genes.1.to.150	85,78%	79,13%
genes.1.to.151	85,67%	79,14%
genes.1.to.152	85,44%	79,21%
genes.1.to.153	85,56%	79,14%
genes.1.to.154	85,56%	79,23%
genes.1.to.200	85,78%	78,77%
genes.1.to.201	85,56%	78,77%
genes.1.to.202	85,78%	78,74%
genes.1.to.203	85,67%	78,69%
genes.1.to.204	86,00%	78,76%
genes.1.to.250	86,11%	78,63%
genes.1.to.251	86,22%	78,64%
genes.1.to.252	86,22%	78,64%
genes.1.to.253	86,00%	78,62%
genes.1.to.254	86,11%	78,64%

genes.1.to.300	86,22%	78,32%
genes.1.to.301	86,00%	78,31%
genes.1.to.302	86,33%	78,27%
genes.1.to.303	86,22%	78,35%
genes.1.to.304	86,78%	78,30%
genes.1.to.350	86,33%	78,29%
genes.1.to.351	86,22%	78,33%
genes.1.to.352	86,00%	78,32%
genes.1.to.353	86,00%	78,38%
genes.1.to.354	85,89%	78,25%
genes.1.to.400	86,89%	78,01%
genes.1.to.401	86,89%	77,98%
genes.1.to.402	86,78%	78,05%
genes.1.to.403	86,44%	78,00%
genes.1.to.404	86,33%	77,95%
genes.1.to.450	87,00%	77,74%
genes.1.to.451	87,33%	77,76%
genes.1.to.452	87,33%	77,87%
genes.1.to.453	87,22%	77,78%
genes.1.to.454	87,11%	77,79%
genes.1.to.500	86,67%	77,45%
genes.1.to.501	86,67%	77,39%
genes.1.to.502	86,56%	77,38%
genes.1.to.503	86,56%	77,36%
genes.1.to.504	86,67%	77,37%
genes.1.to.600	86,44%	77,42%
genes.1.to.601	86,56%	77,43%
genes.1.to.602	86,67%	77,36%
genes.1.to.603	86,67%	77,38%
genes.1.to.604	86,33%	77,36%
genes.1.to.700	86,78%	76,93%
genes.1.to.701	86,56%	76,93%
genes.1.to.702	86,67%	76,84%
genes.1.to.703	86,67%	76,83%
genes.1.to.704	86,67%	76,84%
genes.1.to.800	86,22%	76,36%
genes.1.to.801	86,11%	76,35%
genes.1.to.802	86,56%	76,35%
genes.1.to.803	86,56%	76,36%
genes.1.to.804	86,44%	76,34%
genes.1.to.900	87,67%	76,19%
genes.1.to.901	87,78%	76,18%
genes.1.to.902	87,89%	76,10%
genes.1.to.903	87,89%	76,09%
genes.1.to.904	87,78%	76,13%
genes.1.to.1000	88,00%	75,97%
genes.1.to.1001	88,00%	75,92%
genes.1.to.1002	87,89%	75,95%
genes.1.to.1003	87,89%	75,92%
genes.1.to.1004	88,00%	75,89%
genes.1.to.1200	87,89%	75,96%
genes.1.to.1201	88,00%	75,99%
genes.1.to.1202	88,00%	76,02%
genes.1.to.1203	88,00%	76,02%
genes.1.to.1204	88,00%	76,01%
genes.1.to.1400	88,78%	77,11%
genes.1.to.1401	88,78%	77,18%

genes.1.to.1402	88,89%	77,15%
genes.1.to.1403	89,00%	77,17%
genes.1.to.1404	89,00%	77,18%
genes.1.to.1600	89,00%	77,89%
genes.1.to.1601	88,78%	77,93%
genes.1.to.1602	88,78%	77,88%
genes.1.to.1603	88,89%	77,85%
genes.1.to.1604	88,89%	77,92%
genes.1.to.1800	89,67%	78,14%
genes.1.to.1801	89,56%	78,13%
genes.1.to.1802	89,44%	78,13%
genes.1.to.1803	89,44%	78,16%
genes.1.to.1804	89,44%	78,15%
genes.1.to.2000	89,44%	78,17%
genes.1.to.2001	89,44%	78,17%
genes.1.to.2002	89,44%	78,14%
genes.1.to.2003	89,44%	78,10%
genes.1.to.2004	89,22%	78,15%

B) *100 best performing probesets and genes, which they detect sorted according to the importance in the contribution to distinguish cetuximab responders and non-responders in the xenografts used in the treatment experiment; mRNA expression analysis performed on the U133 Plus 2.0 GeneChip*

No	**Probeset_Affymetrix**	**Gene symbol**	**Gene name**	**gene.index**	**frequency**
1	1558685_a_at	LOC158960	hypothetical protein BC009467	372	100,00%
2	1569583_at	EREG	epiregulin	509	100,00%
3	200940_s_at	RERE	arginine-glutamic acid dipeptide (RE) repeats	931	100,00%
4	1568597_at	LOC646762	hypothetical LOC646762	476	100,00%
5	1553581_s_at	SFRS12IP1	SFRS12-interacting protein 1; family with sequence similarity 159, member B	67	100,00%
6	200636_s_at	PTPRF	protein tyrosine phosphatase, receptor type, F	656	100,00%
7	1557285_at	AREGB	amphiregulin; amphiregulin B	318	100,00%
8	200920_s_at	BTG1	B-cell translocation gene 1, anti-proliferative	917	100,00%
9	200698_at	KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	712	100,00%
10	1555962_at	B3GNT7	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7	258	100,00%
11	1553015_a_at	RECQL4	RecQ protein-like 4	46	100,00%
12	200976_s_at	TAX1BP1	Tax1 (human T-cell leukemia virus type I) binding protein 1	965	100,00%
13	200921_s_at	BTG1	B-cell translocation gene 1, anti-proliferative	918	100,00%

14	1554608_at	TGOLN2	trans-golgi network protein 2	168	100,00%
15	1560916_a_at	LOC100133317	dpy-19-like 1 (C. elegans); similar to hCG1645499	427	100,00%
16	1552519_at	ACVR1C	activin A receptor, type IC	27	100,00%
17	1558412_at	LOC113230	hypothetical protein LOC113230	361	100,00%
18	1559957_a_at	LOC642852	hypothetical LOC642852	409	100,00%
19	200745_s_at	GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1	756	100,00%
20	200637_s_at	PTPRF	protein tyrosine phosphatase, receptor type, F	657	100,00%
21	1552954_at	C5ORF17	chromosome 5 open reading frame 17	45	100,00%
22	1552628_a_at	HERPUD2	HERPUD family member 2	33	100,00%
23	1553972_a_at	CBS	cystathionine-beta-synthase	98	100,00%
24	200612_s_at	AP2B1	adaptor-related protein complex 2, beta 1 subunit	632	100,00%
25	1555963_x_at	B3GNT7	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7	259	100,00%
26	201032_at	BLCAP	bladder cancer associated protein	1016	100,00%
27	1556242_a_at	LOC727872	hypothetical protein LOC727872	280	100,00%
28	200062_s_at	RPL30	ribosomal protein L30	577	100,00%
29	200699_at	KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	713	100,00%
30	1554679_a_at	LAPTM4B	lysosomal protein transmembrane 4 beta	173	100,00%
31	200647_x_at	EIF3CL	eukaryotic translation initiation factor 3, subunit C-like	666	100,00%
32	200638_s_at	YWHAZ	tyrosine 3-monooxygenase/tryptophan	658	100,00%
33	201066_at	CYC1	cytochrome c-1	1044	100,00%
34	200989_at	HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	977	100,00%
35	1554021_a_at	ZNF12	postmeiotic segregation increased 2-like 3; zinc finger protein 12	116	100,00%
36	1553589_a_at	PDZK1IP1	PDZK1 interacting protein 1	70	100,00%
37	1557207_s_at	LOC283177	hypothetical protein LOC283177	315	100,00%
38	200640_at	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	660	100,00%
39	1554576_a_at	ETV4	ets variant 4	163	100,00%

40	1556228_a_at	VCPIP1	valosin containing protein (p97)/p47 complex interacting protein 1	279	100,00%
41	200931_s_at	VCL	vinculin	925	100,00%
42	1569110_x_at	LOC728613	programmed cell death 6 pseudogene	500	100,00%
43	200090_at	FNTA	farnesyltransferase, CAAX box, alpha	604	100,00%
44	1553960_at	SNX21	sorting nexin family member 21	96	100,00%
45	1553709_a_at	PRPF38A	PRP38 pre-mRNA processing factor 38 (yeast) domain containing A	80	100,00%
46	1568983_a_at	-	-	496	100,00%
47	201058_s_at	MYL9	myosin, light chain 9, regulatory	1037	100,00%
48	1561421_a_at	MUC3B	mucin 3B, cell surface associated	431	100,00%
49	1552845_at	CLDN15	claudin 15	42	100,00%
50	200975_at	PPT1	palmitoyl-protein thioesterase 1	964	100,00%
51	200608_s_at	RAD21	RAD21 homolog (S. pombe)	628	100,00%
52	200053_at	SPAG7	sperm associated antigen 7	568	100,00%
53	200073_s_at	HNRNPD	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	588	100,00%
54	1568807_a_at	NDFIP2	Nedd4 family interacting protein 2	492	100,00%
55	1555370_a_at	CAMTA1	calmodulin binding transcription activator 1	203	100,00%
56	1554436_a_at	REG4	regenerating islet-derived family, member 4	149	100,00%
57	1552691_at	ARL11	ADP-ribosylation factor-like 11	35	100,00%
58	200977_s_at	TAX1BP1	Tax1 (human T-cell leukemia virus type I) binding protein 1	966	100,00%
59	1556429_a_at	WDR67	WD repeat domain 67	285	100,00%
60	1555765_a_at	GNG4	guanine nucleotide binding protein (G protein), gamma 4	220	100,00%
61	201034_at	ADD3	adducin 3 (gamma)	1018	100,00%
62	1567014_s_at	NFE2L2	nuclear factor (erythroid-derived 2)-like 2	466	100,00%
63	200948_at	MLF2	myeloid leukemia factor 2	939	100,00%
64	200639_s_at	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	659	100,00%

65	1558540_s_at	SLC2A11	solute carrier family 2 (facilitated glucose transporter), member 11	366	100,00%
66	200661_at	CTSA	cathepsin A	680	100,00%
67	1558048_x_at	-	-	341	100,00%
68	1559910_at	PID1	phosphotyrosine interaction domain containing 1	407	100,00%
69	200620_at	TMEM59	transmembrane protein 59	640	100,00%
70	200691_s_at	HSPA9	heat shock 70kDa protein 9 (mortalin)	705	100,00%
71	200903_s_at	AHCY	adenosylhomocysteinase	901	100,00%
72	1555841_at	TMEFF1	transmembrane protein with EGF-like and two follistatin-like domains 1	230	100,00%
73	1568603_at	CADPS	Ca++-dependent secretion activator	478	100,00%
74	1555888_at	UBR5	similar to E3 ubiquitin protein ligase, HECT domain containing, 1	245	100,00%
75	200807_s_at	HSPD1P6	heat shock 60kDa protein 1 (chaperonin) pseudogene 6	813	100,00%
76	200675_at	CD81	CD81 molecule	691	100,00%
77	200641_s_at	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	661	100,00%
78	200837_at	BCAP31	B-cell receptor-associated protein 31	840	100,00%
79	1560297_at	HM13	histocompatibility (minor) 13	419	100,00%
80	1554003_at	RGNEF	Rho-guanine nucleotide exchange factor	112	100,00%
81	200973_s_at	TSPAN3	tetraspanin 3	963	100,00%
82	1555797_a_at	ARPC5	actin related protein 2/3 complex, subunit 5, 16kDa	224	100,00%
83	200645_at	GABARAP	GABA(A) receptor-associated protein	665	100,00%
84	1558154_at	LLGL2	lethal giant larvae homolog 2 (Drosophila)	348	100,00%
85	200936_at	RPL8P2	ribosomal protein L8 pseudogene 2	929	100,00%
86	1556047_s_at	MAGEE1	melanoma antigen family E, 1	268	100,00%
87	1557521_a_at	BLCAP	bladder cancer associated protein	328	100,00%
88	1553743_at	FAM119A	family with sequence similarity 119, member A	82	100,00%
89	201110_s_at	THBS1	thrombospondin 1	1082	100,00%
90	200039_s_at	PSMB2	proteasome (prosome, macropain) subunit, beta type, 2	558	100,00%

91	1555890_at	OR2A20P	olfactory receptor, family 2, subfamily A, member 9 pseudogene	247	100,00%
92	200063_s_at	LOC399804	pseudogene 21; hypothetical LOC100131044; similar to nucleophosmin 1	578	100,00%
93	1557522_x_at	BLCAP	bladder cancer associated protein	329	100,00%
94	200702_s_at	DDX24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	716	100,00%
95	200635_s_at	PTPRF	protein tyrosine phosphatase, receptor type, F	655	100,00%
96	201109_s_at	THBS1	thrombospondin 1	1081	100,00%
97	200926_at	RPS23	ribosomal protein S23	922	100,00%
98	1566557_at	FLJ90757	hypothetical LOC440465	461	100,00%
99	1555427_s_at	SYNCRIP	synaptotagmin binding, cytoplasmic RNA interacting protein	204	100,00%
100	200821_at	LAMP2	lysosomal-associated membrane protein 2	826	100,00%

Tab.S11. A) *Performance of the miRNA signature in terms of sensitivity and specificity during discovery on the xenografts used in the treatment experiment, sorted according to the number of probesets included in the signature; miRNA expression analysis performed on the Affymetrix GeneChip miRNA 2.0*

Genes No included in the signature	**Sensitivity**	**Specificity**
genes.1.to.3	50,45%	54,73%
genes.1.to.4	51,59%	56,18%
genes.1.to.5	51,82%	57,50%
genes.1.to.6	52,95%	58,50%
genes.1.to.7	54,55%	58,36%
genes.1.to.10	53,86%	61,18%
genes.1.to.11	54,43%	61,91%
genes.1.to.12	55,00%	61,27%
genes.1.to.13	57,61%	60,55%
genes.1.to.14	56,93%	61,09%
genes.1.to.20	58,30%	60,55%
genes.1.to.21	58,98%	61,23%
genes.1.to.22	60,34%	61,27%
genes.1.to.23	60,91%	62,68%
genes.1.to.24	61,14%	63,09%
genes.1.to.30	61,48%	63,86%
genes.1.to.31	61,36%	64,36%
genes.1.to.32	61,14%	64,09%
genes.1.to.33	60,91%	63,73%
genes.1.to.34	60,91%	63,86%
genes.1.to.40	61,02%	63,50%
genes.1.to.41	61,59%	63,68%
genes.1.to.42	61,36%	64,05%
genes.1.to.43	60,57%	63,27%
genes.1.to.44	60,80%	63,36%
genes.1.to.50	61,70%	63,41%

genes.1.to.51	62,84%	63,68%
genes.1.to.52	62,84%	63,77%
genes.1.to.53	62,95%	64,27%
genes.1.to.54	63,41%	64,36%
genes.1.to.100	66,70%	66,50%
genes.1.to.101	66,25%	66,68%
genes.1.to.102	66,02%	66,45%
genes.1.to.103	66,02%	66,50%
genes.1.to.104	66,02%	66,77%
genes.1.to.150	68,07%	67,91%
genes.1.to.151	68,07%	67,50%
genes.1.to.152	67,95%	67,55%
genes.1.to.153	67,84%	67,68%
genes.1.to.154	68,41%	67,77%
genes.1.to.200	71,02%	68,23%
genes.1.to.201	71,14%	68,32%
genes.1.to.202	71,48%	68,18%
genes.1.to.203	70,80%	68,23%
genes.1.to.204	71,02%	68,23%
genes.1.to.250	72,95%	67,55%
genes.1.to.251	72,73%	67,32%
genes.1.to.252	72,73%	67,36%
genes.1.to.253	72,73%	67,45%
genes.1.to.254	72,50%	67,50%
genes.1.to.300	74,89%	67,05%
genes.1.to.301	75,57%	67,32%
genes.1.to.302	75,57%	67,36%
genes.1.to.303	74,89%	67,45%
genes.1.to.304	74,66%	67,59%
genes.1.to.350	75,34%	68,00%
genes.1.to.351	75,57%	67,95%
genes.1.to.352	75,68%	68,05%
genes.1.to.353	75,91%	67,91%
genes.1.to.354	75,91%	67,95%
genes.1.to.400	75,91%	67,73%
genes.1.to.401	75,91%	67,73%
genes.1.to.402	75,80%	67,82%
genes.1.to.403	75,57%	67,82%
genes.1.to.404	75,57%	67,73%
genes.1.to.450	76,59%	67,68%
genes.1.to.451	76,48%	67,73%
genes.1.to.452	76,59%	67,59%
genes.1.to.453	76,59%	67,55%
genes.1.to.454	76,59%	67,68%
genes.1.to.500	77,05%	66,36%
genes.1.to.501	77,16%	66,23%
genes.1.to.502	77,05%	66,23%
genes.1.to.503	77,05%	66,23%
genes.1.to.504	76,93%	66,36%
genes.1.to.600	78,75%	65,45%
genes.1.to.601	78,86%	65,50%
genes.1.to.602	78,98%	65,41%
genes.1.to.603	78,86%	65,36%
genes.1.to.604	78,98%	65,36%
genes.1.to.700	80,45%	64,45%
genes.1.to.701	80,45%	64,41%
genes.1.to.702	80,23%	64,41%

genes.1.to.703	80,11%	64,50%
genes.1.to.704	80,11%	64,41%
genes.1.to.800	80,34%	63,86%
genes.1.to.801	80,68%	63,82%
genes.1.to.802	80,80%	63,68%
genes.1.to.803	80,91%	63,95%
genes.1.to.804	80,57%	63,86%
genes.1.to.900	80,68%	63,59%
genes.1.to.901	80,57%	63,59%
genes.1.to.902	80,34%	63,59%
genes.1.to.903	80,34%	63,73%
genes.1.to.904	80,57%	63,73%
genes.1.to.1000	80,68%	63,55%
genes.1.to.1001	80,68%	63,50%
genes.1.to.1002	80,68%	63,55%
genes.1.to.1003	80,68%	63,55%
genes.1.to.1004	80,68%	63,36%
genes.1.to.1200	81,14%	63,68%
genes.1.to.1201	81,25%	63,82%
genes.1.to.1202	81,25%	63,82%
genes.1.to.1203	81,36%	63,73%
genes.1.to.1204	81,48%	63,68%
genes.1.to.1400	82,27%	63,86%
genes.1.to.1401	82,27%	63,77%
genes.1.to.1402	82,16%	63,82%
genes.1.to.1403	82,16%	63,82%
genes.1.to.1404	82,27%	63,91%

B) *100 best performing probesets sorted according to the importance in the contribution to distinguish cetuximab responders and non-responders in xenografts used in the treatment experiment; miRNA expression analysis performed on the Affymetrix GeneChip miRNA 2.0*

	Probeset_Affymetrix	**gene.index**	**frequency**
1	rno-miR-125a-5p_st	1136	100%
2	bta-let-7e_st	61	100%
3	hsa-miR-224_st	550	100%
4	xtr-miR-125a_st	1361	100%
5	rno-miR-146b_st	1147	100%
6	hsa-miR-125a-5p_st	481	100%
7	mmu-miR-125a-5p_st	844	100%
8	rno-let-7e_st	1129	100%
9	fru-miR-125a_st	321	100%
10	bta-miR-125a_st	70	100%
11	dre-miR-125a_st	257	100%
12	bta-miR-151_st	79	100%
13	cfa-miR-125a_st	147	100%
14	mml-miR-125a-5p_st	721	100%
15	ggo-miR-224_st	434	100%
16	hsa-miR-99b_st	615	100%
17	hsa-miR-720_st	602	100%
18	ssc-miR-99b_st	1274	100%
19	mmu-miR-720_st	940	100%
20	ppa-miR-30d_st	1030	100%
21	tni-miR-10b_st	1281	100%
22	cfa-let-7e_st	139	100%
23	ppa-miR-224_st	1020	100%

24	bta-miR-151-star_st	80	100%
25	mml-miR-151-5p_st	735	100%
26	mne-miR-224_st	974	100%
27	rno-miR-99b_st	1219	100%
28	cfa-miR-30a_st	192	100%
29	ptr-miR-224_st	1108	100%
30	mne-miR-30d_st	984	100%
31	hsa-miR-182_st	517	100%
32	hsa-let-7e_st	465	100%
33	mml-let-7e_st	709	100%
34	mml-miR-99b_st	824	100%
35	ssc-miR-224_st	1268	100%
36	cfa-miR-224_st	181	100%
37	hsa-miR-143_st	499	100%
38	cfa-miR-151_st	157	100%
39	rno-miR-30d_st	1198	100%
40	cfa-miR-99b_st	214	100%
41	U48_st	1330	100%
42	mmu-miR-151-5p_st	857	100%
43	hsa-miR-151-3p_st	506	100%
44	mmu-miR-421_st	921	100%
45	ggo-miR-195_st	424	100%
46	fru-miR-16_st	325	100%
47	tni-miR-125a_st	1282	100%
48	mml-miR-224_st	774	100%
49	rno-miR-342-3p_st	1204	100%
50	dre-miR-30d_st	303	100%
51	mml-miR-151-3p_st	734	100%
52	ppy-miR-224_st	1068	100%
53	hsa-miR-483-5p_st	584	100%
54	rno-miR-361_st	1206	100%
55	bta-miR-30d_st	118	100%
56	ppa-miR-195_st	1011	100%
57	hsa-miR-151-5p_st	507	100%
58	mmu-miR-361_st	916	100%
59	mmu-miR-99b_st	947	100%
60	ptr-miR-30d_st	1119	100%
61	mmu-let-7e_st	829	100%
62	bta-miR-150_st	78	100%
63	cfa-miR-30d_st	195	100%
64	mmu-miR-195_st	875	100%
65	fru-miR-30d_st	352	100%
66	hsa-miR-339-5p_st	570	100%
67	dre-miR-27b_st	301	100%
68	cfa-miR-320_st	197	100%
69	mml-miR-652_st	811	100%
70	dre-miR-183_st	273	100%
71	rno-miR-224_st	1184	100%
72	bta-miR-99b_st	132	100%
73	mmu-miR-31_st	910	100%
74	rno-miR-195_st	1166	100%
75	AFFX-CreX-5_at	3	100%
76	mdo-miR-143_st	668	100%
77	cel-miR-72_st	136	100%
78	mml-miR-320_st	789	100%
79	bta-miR-139_st	74	100%
80	ssc-miR-145_st	1258	100%

81	mmu-miR-145_st	853	100%
82	rno-miR-151_st	1149	100%
83	gga-miR-10b_st	365	100%
84	rno-miR-652_st	1213	100%
85	bta-let-7d_st	60	100%
86	mmu-miR-143_st	852	100%
87	mdo-miR-10a_st	664	100%
88	hsa-miR-886-5p_st	608	100%
89	hsa-miR-320c_st	568	100%
90	hsa-miR-423-3p_st	579	100%
91	hsa-miR-320b_st	567	100%
92	mml-miR-10a_st	717	100%
93	hsa-miR-30d_st	564	100%
94	bta-miR-320_st	121	100%
95	cfa-miR-361_st	201	100%
96	mmu-miR-483_st	925	100%
97	ggo-miR-30d_st	443	100%
98	hsa-miR-1308_st	492	100%
99	bta-miR-126_st	72	100%
100	rno-miR-205_st	1176	100%

Tab.S12. *The largest randomized clinical trials testing efficacy of anti-EGFR mAb (cetuximab or panitumumab) and their effect on the survival of the patients*

Reference	**Treatment regimens**	**Study population**	**Number of patients**	**Response Rate**	**Survival benefit**	**Findings**
Cunningham et al., 2004	Cetuximab vs. Cetuximab + irinotecan	Irinotecan refractory, Phase III, mCRC	329	11% vs. 23%	Median survival time = 6.9 months (monotherapy) vs.8.6 (combination therapy) (p=0.48).	No correlation between the expression of EGFR (IHC) and response to cetuximab
Monotherapy						
Giusti et al., 2007	Best supportive care (BSC) vs. Best supportive care + panitumumab	Chemorefractory, mCRC	463	0% vs. 8%	Median PFS = 51 (BSC) vs. 56 days (panitumumab)	No difference in OS between the two study arms
Amado et al., 2008	Best supportive care vs. Best supportive care + panitumumab	Chemorefractory, Phase III, mCRC	427	0% vs. 17% (KRAS WT) 0%; (KRAS mut)	Median PFS = 12.3 weeks (panitumumab) vs. 7.3 weeks (BSC) in the WT KRAS group	WT KRAS - required for panitumumab efficacy; KRAS mutations found in 43% pts
Karapetis et al., 2008	Best supportive care vs. Best supportive care + cetuximab; NCIC-017	Chemorefractory, Phase III, mCRC	394	0% vs. 12.8%(KRAS WT); 1.2% (KRAS mut)	Median OS= 9.5(cetuximab) vs. 4.8 months (BSC) (P<0.001); Median PFS= 3.7 months vs. 1.9 months (P<0.001) in the WT KRAS group	KRAS mutations found in 42% pts - confirmed biomarker of resistance
Van Cutsem	Best supportive care vs. Best supportive care + panitumumab	Chemorefractory, phase III, mCRC	463 (232 vs. 231)	0% vs. 10%	Median PFS = 8 weeks (panitumumab) vs. 7.3 weeks (BSC) No difference was observed in OS.	Panitumumab significantly improved PFS with manageable toxicity in patients with chemorefractory colorectal cancer.
Jonker	Best supportive care vs. Best supportive care + cetuximab	Chemorefractory, mCRC, mCRC	572 (285 vs. 287)	0% vs. 8%	Median OS= 6.1 months (cetuximab) vs. 4.6 months (BSC)	Cetuximab improves OS and PFS in chemorefractory pts.
Chemonaive patients – first-line therapy						
Bokemeyer et al., 2009	FOLFOX4 vs. FOLFOX4 + cetuximab; OPUS	Chemonaïve, first line, Phase II, mCRC	315 (KRAS status assessed in 233)	36% vs. 46% (ORR)	Lower risk of disease progression under cetuximab (P = .0163) compared with FOLFOX-4 alone in the KRAS WT group	Increased ORR under cetuximab in KRAS WT : 61% v 37%, No PFS benefit
Van Cutsem et al., 2009	FOLFIRI vs. FOLFIRI + cetuximab; CRYSTAL	Chemonaïve, first line, Phase III, mCRC	599 vs. 599 (KRAS status assessed	39% vs. 47% (ORR)	PFS hazard ratio in 0.85 (95% confidence interval [CI], 0.72 to 0.99; P=0.048) (+cetuximab) No difference in OS (P=0.31).	KRAS mutation correlates with response (P=0.03) but not with PFS (P=0.07) or OS (P=0.44); cetuximab + FOLFIRI reduced the risk of progression by 15% as

			in 540)			compared with FOLFIRI alone
Combination therapies of anti-EGFR and bevacizumab						
Tol et al.2009	CAPOX, +bevacizumab vs. CAPOX, +bevacizumab + cetuximab	Chemonaïve, first line, mCRC	378 vs. 377	50% vs. 53% (ORR)	Median PFS= 10.7 (CAPOX, +bevacizumab) vs. 9.4 months (CAPOX, +bevacizumab + cetuximab) (P=0.01) Median OS= 20.3 months vs.19.4 (P = 0.16)	PFS – decreased, toxicity - increased after the addition of cetuximab to capecitabine, oxaliplatin, and bevacizumab; KRAS mutation status - predictor of outcome in the cetuximab group
Hecht, 2009	FOLFOX/FOLFIRI + bevacizumab vs. FOLFOX/FOLFIRI + bevacizumab + panitumumab	First line, phase IIIB, mCRC	1053 (525 vs. 528)	46% vs. 48%	Median PFS=11.4 (control arm) vs. 10.0 (+panitumumab) Median OS= 24.5 (control arm) vs. 19.4 (+panitumumab)	PFS – decreased, toxicity - increased after the addition of panitumumab to bevacizumab and oxaliplatin- or irinotecan-based
Saltz et al.	Cetuximab + bevacizumab vs. Cetuximab + bevacizumab + irinotecan	Chemonaïve, mCRC	40 vs. 43	20% vs. 37%	Time to tumour progression (TTP)= 7.3 months (cetuximab + bevacizumab) vs. 4.9 months (cetuximab + bevacizumab + irinotecan)	The activity of bevacizumab + cetuximab (+/-irinotecan) is favorable when compared with historical controls of cetuximab or cetuximab/irinotecan
Combined with chemotherapy						
Sobrero et al. 2008	Irinotecan vs. Irinotecan + cetuximab	Chemorefractory, second line, mCRC	1298	4% vs. 16%	Median OS = 10.0 months (irinotecan) vs. 10.7 months (cetuximab + irinotecan) Median PFS= 2.6 months vs. 4.0 months (P <or= .0001)	Cetuximab and irinotecan improved PFS and ORR. OS was similar between study groups,
Peeters et al. 2010	FOLFIRI vs. FOLFIRI + cetuximab	Chemorefractory, mCRC	1186 (KRAS status assessed in 1083)	10% vs. 35%	Median PFS 5.9 months (panitumumab + FOLFIRI) vs. 3.9 months (FOLFIRI). A nonsignificant trend toward increased OS was observed.	Panitumumab plus FOLFIRI significantly improved PFS and is well-tolerated as second-line treatment in patients with WT KRAS mCRC.
Maughan et al. 2011	Chemotherapy vs. chemotherapy + cetuximab; COIN trial	Chemonaive, mCRC	1630 (815 vs. 815) (Mutation status assessed in 1316)	57% vs. 64% (ORR)(p=0.049)	OS- no difference between treatment groups in KRAS WT OS= 17.9 months (control arm) vs.17.0 months (+ cetuximab) (p=0.67). Median PFS= 8.6 months (control arm) vs. 8.6 months (+ cetuximab) (p=0·60).	Cetuximab increases ORR in KRAS WT pts, no PFS or OS benefit observed OS differs by somatic mutation status irrespective of treatment: BRAF mutant – 9 months; KRAS mutant – 14 months; WT- 20 months
Trials in stage III CRC						
Alberts et al. 2011	FOLFOX6 vs. FOLFOX6 + cetuximab; trial N0147	Chemonaive, stage III	1.760 pts of stage III CRC wild-type in KRAS	-	3-yr DFS favored FOLFOX alone (HR 1.18, 95% CI 0.92-1.52; p=0.33); trial closed	Addition of cetuximab to FOLFOX6 was of no benefit in resected KRAS wild-type pts of stage III CRC

Goldberg et al. 2010	FOLFOX6 vs. FOLFOX6 + cetuximab; trial N0147	Chemonaive, stage III	658 pts of stage III CRC mutated in KRAS	-	3-yr DFS favored FOLFOX alone (HR 1.48, 95% CI 1.08-2.03; p=0.02); OS favored FOLFOX alone (HR 1.67, CI 1.00-2.80; p=0.07)	Addition of cetuximab to FOLFOX6 resulted in impaired DFS and a trend toward impaired OS in KRAS mutated CRC pts of stage III